# TREATMENT OF NSCLC PATIENTS REFRACTORY FOR ANTI-PD-1 ANTIBODY

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims priority to U.S. Provisional Patent Application No. 62/756,025, filed on November 5, 2018, and U.S. Provisional Patent Application No. 62/903,627, filed on September 20, 2019, which are hereby incorporated by reference in their entirety.

## BACKGROUND OF THE INVENTION

**[0002]** Treatment of bulky, refractory cancers using adoptive transfer of tumor infiltrating lymphocytes (TILs) represents a powerful approach to therapy for patients with poor prognoses. Gattinoni, *et al.*, *Nat. Rev. Immunol.* **2006,** *6,* 383-393. A large number of TILs are required for successful immunotherapy, and a robust and reliable process is needed for commercialization. This has been a challenge to achieve because of technical, logistical, and regulatory issues with cell expansion. IL-2-based TIL expansion followed by a "rapid expansion process" (REP) has become a preferred method for TIL expansion because of its speed and efficiency. Dudley, *et al.*, *Science* **2002,** *298,* 850-54; Dudley, *et al.*, *J. Clin. Oncol.* **2005,** *23,* 2346-57; Dudley, *et al.*, *J. Clin. Oncol.* **2008**, *26,* 5233-39; Riddell, *et al.*, *Science* **1992,** *257,* 238-41; Dudley, *et al.*, *J. Immunother.* **2003**, *26,* 332-42. REP can result in a 1,000-fold expansion of TILs over a 14-day period, although it requires a large excess (*e.g.*, 200-fold) of irradiated allogeneic peripheral blood mononuclear cells (PBMCs, also known as mononuclear cells (MNCs)), often from multiple donors, as feeder cells, as well as anti-CD3 antibody (OKT3) and high doses of IL-2. Dudley, *et al., J. Immunother.* **2003,** *26,* 332-42. TILs that have undergone an REP procedure have produced successful adoptive cell therapy following host immunosuppression in patients with melanoma. Current infusion acceptance parameters rely on readouts of the composition of TILs (*e.g.*, CD28, CD8, or CD4 positivity) and on fold expansion and viability of the REP product.

**[0003]** Current TIL manufacturing and treatment processes are limited by length, cost, sterility concerns, and other factors described herein such that the potential to treat patients which are refractory to anti-PD1 and as such have been severly limited. There is an urgent need to provide TIL manufacturing processes and therapies based on such processes that are

appropriate for use in treating patients for whom very few or no viable treatment options remain. The present invention meets this need by providing a shortened manufacturing process for use in generating TILs which can then be employed in the treatment of non-small cell lung carcinoma (NSCLC) patients whom are refractory to anti-PD-1 treatment.

## BRIEF SUMMARY OF THE INVENTION

**[0004]** The present invention provides improved and/or shortened methods for expanding TILs and producing therapeutic populations of TILs for use in treatment of non-small cell lung carcinoma (NSCLC) patients whom are refractory to anti-PD-1 treatment.

**[0005]** The present invention provides a method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor infiltrating lymphocytes (TILs) comprising the steps of:

(a) obtaining and/or receiving a first population of TILs from surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells from a NSCLC tumor in a patient;

(c) contacting the tumor fragments with a first cell culture medium;

(d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

(e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

(f) harvesting the third population of TILs; and

(g) administering a therapeutically effective portion of the third population of TILs to a patient with the NSCLC;

wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

**[0006]** In some embodiments, "obtaining" indicates the TILs employed in the method and/or process can be derived directly from the sample (including from a surgical resection, needle biopsy, core biopsy, small biopsy, or other sample) as part of the method and/or process steps. In some embodiments, "receiving" indicates the TILs employed in the method and/or process can be derived indirectly from the sample (including from a surgical resection, needle biopsy, core biopsy, small biopsy, or other sample) and then employed in the method and/or process, (for example, where step (a) begins will TILs that have already been derived from the sample by a separate process not included in part (a), such TILs could be refered to as "received").

**[0007]** In some embodiments, the first population of TILs comprises a multilesional sampling method.

**[0008]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 and/or anti-PD-L2 antibody.

**[0009]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

**[0010]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent.

**[0011]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

**[0012]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

**[0013]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

**[0014]** In some embodiments, the refractory NSCLC has low expression of PD-L1.

**[0015]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

**[0016]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

**[0017]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

**[0018]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

**[0019]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

**[0020]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

**[0021]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

**[0022]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

**[0023]** In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

**[0024]** In some embodiments, the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

**[0025]** In some embodiments, the refractory NSCLC is refractory to an anti-PD-1 antibody or an anti-PD-L1 selected from the group consisting of nivolumab, pembrolizumab, ipilimumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

**[0026]** In some embodiments, the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

**[0027]** In some embodiments, the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

**[0028]** In some embodiments, the refractory NSCLC is refractory to ipilimumab or a biosimilar thereof.

**[0029]** In some embodiments, the refractory NSCLC is refractory to ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

**[0030]** In some embodiments, the refractory NSCLC is refractory to ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

**[0031]** In some embodiments, the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

**[0032]** In some embodiments, the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

**[0033]** In some embodiments, the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

**[0034]** In some embodiments, the initial expansion is performed over a period of 21 days or less.

**[0035]** In some embodiments, the initial expansion is performed over a period of 14 days or less.

**[0036]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

**[0037]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL in the second cell culture medium.

**[0038]** In some embodiments, the initial expansion is performed using a gas permeable container.

**[0039]** In some embodiments, the rapid expansion is performed using a gas permeable container.

**[0040]** In some embodiments, the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

**[0041]** In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

**[0042]** In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

**[0043]** In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

**[0044]** In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

**[0045]** In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

**[0046]** In some embodiments, the invention provides a method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor infiltrating lymphocytes (TILs) comprising the steps of:

(a) resecting a tumor from a patient, the tumor comprising a first population of TILs;

(b) fragmenting the tumor into tumor fragments;

(c) contacting the tumor fragments with a first cell culture medium;

(d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

(e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

(f) harvesting the third population of TILs; and

(g) administering a therapeutically effective portion of the third population of TILs to a

patient with the cancer;

wherein the cancer is refractory to treatment with an anti-PD-1 antibody.

**[0047]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

**[0048]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

**[0049]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent.

**[0050]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

**[0051]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

**[0052]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

**[0053]** In some embodiments, the refractory NSCLC has low expression of PD-L1.

**[0054]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

**[0055]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

**[0056]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

**[0057]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

**[0058]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

**[0059]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

**[0060]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

**[0061]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

**[0062]** In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

**[0063]** In some embodiments, the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

**[0064]** In some embodiments, the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

**[0065]** In some embodiments, the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

**[0066]** In some embodiments, the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

**[0067]** In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

**[0068]** In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

**[0069]** In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

**[0070]** In some embodiments, the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

**[0071]** In some embodiments, the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

**[0072]** In some embodiments, the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

**[0073]** In some embodiments, the initial expansion is performed over a period of 21 days or less.

**[0074]** In some embodiments, the initial expansion is performed over a period of 14 days or less.

**[0075]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

**[0076]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL in the second cell culture medium.

**[0077]** In some embodiments, the initial expansion is performed using a gas permeable container.

**[0078]** In some embodiments, the rapid expansion is performed using a gas permeable container.

**[0079]** In some embodiments, the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

**[0080]** In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

**[0081]** In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

**[0082]** In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

**[0083]** In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

**[0084]** In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

**[0085]** In some embodiments, the invention provides a method for treating a subject with non-small cell lung carcinoma (NSCLC), wherein the cancer is refractory to treatment with an anti-PD-1 antibody, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject into multiple tumor fragments;

(b) adding the tumor fragments into a closed system;

(c) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11~~3-14~~ days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11~~7-14~~ days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;

(e) harvesting therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system; and

(f) transferring the harvested TIL population from step (e) to an infusion bag, wherein the transfer from step (e) to (f) occurs without opening the system;

(g) cryopreserving the infusion bag comprising the harvested TIL population from step (f) using a cryopreservation process; and

(h) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

**[0086]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

**[0087]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

**[0088]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent.

**[0089]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

**[0090]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

**[0091]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

**[0092]** In some embodiments, the refractory NSCLC has low expression of PD-L1.

**[0093]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

**[0094]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

**[0095]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

**[0096]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1.

**[0097]** In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

**[0098]** In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

**[0099]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

**[00100]** In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

**[00101]** In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

**[00102]** In some embodiments, the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

**[00103]** In some embodiments, the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

**[00104]** In some embodiments, the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

**[00105]** In some embodiments, the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

**[00106]** In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

**[00107]** In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

**[00108]** In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

**[00109]** In some embodiments, the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

**[00110]** In some embodiments, the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

**[00111]** In some embodiments, the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

**[00112]** In some embodiments, the initial expansion is performed over a period of 21 days or less.

**[00113]** In some embodiments, the initial expansion is performed over a period of 14 days or less.

**[00114]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

**[00115]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL in the second cell culture medium.

**[00116]** In some embodiments, the initial expansion is performed using a gas permeable container.

**[00117]** In some embodiments, the rapid expansion is performed using a gas permeable container.

**[00118]** In some embodiments, the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

**[00119]** In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

**[00120]** In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

**[00121]** In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

**[00122]** In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

**[00123]** In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

**[00124]** In some embodiments, the NSCLC is refractory to a combination treatment comprising an anti-PD-1 and chemotherapeutic agent.

**[00125]** In some embodiments, the anti-PD-1 or the anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

**[00126]** In some embodiments, the antiPD-1 is pembrolizumab.

**[00127]** In some embodiments, the chemotherapeutic agent(s) is a platinum doublet chemotherapeutic agent.

**[00128]** In some embodiments, the platinum doublet therapy comprises:

i) a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin,

ii) and a second chemotherapeutic agent selected from the group consisting of vinorelbine, gemcitabine and a taxane (including for example, paclitaxel, docetaxel or nab-paclitaxel).

**[00129]** In some embodiments, the chemotherapeutic agent is in combination with pemetrexed.

**[00130]** In some embodiments, the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, and cisplatin.

**[00131]** In some embodiments, the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, cisplatin, nivolumab, and ipilimumab.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[00132]** **Figure 1:** Exemplary Process 2A chart providing an overview of Steps A through F.

**[00133]** **Figure 2:** Process Flow Chart of Process 2A.

**[00134]** **Figure 3:** Shows a diagram of an embodiment of a cryopreserved TIL exemplary manufacturing process (~22 days).

**[00135]** **Figure 4:** Shows a diagram of an embodiment of process 2A, a 22-day process for TIL manufacturing.

**[00136]** **Figure 5:** Comparison table of Steps A through F from exemplary embodiments of process 1C and process 2A.

**[00137]** **Figure 6:** Detailed comparison of an embodiment of process 1C and an embodiment of process 2A.

**[00138]** **Figure 7:** Exemplary GEN 3 type process for NSCLC tumors.

**[00139]** **Figure 8A-8B: A)** Shows a comparison between the 2A process (approximately 22-day process) and an embodiment of the Gen 3 process for TIL manufacturing (approximately 14-days to 16-days process). **B)** Exemplary Process Gen3 chart providing an overview of Steps A through F (approximately 14-days to 16-days process). **C)** Chart providing three exemplary Gen 3 processes with an overview of Steps A through F (approximately 14-days to 16-days process) for each of the three process variations.

**[00140]** **Figure 9:** Provides an experimental flow chart for comparability between GEN 2 (process 2A) versus GEN 3.

**[00141]** **Figure 10A-10C: A)** L4054 - Phenotypic characterization on TIL product on Gen 2 and Gen 3 process. **B)** L4055-Phenotypic characterization on TIL product on Gen 2 and Gen 3 process. **C)** M1085T-Phenotypic characterization on TIL product on Gen 2 and Gen 3 process.

**[00142]** **Figure 11A-11C**: **A)** L4054 – Memory markers analysis on TIL product from the Gen 2 and Gen 3 processes. **B)** L4055 – Memory markers analysis on TIL product from the

Gen 2 and Gen 3 processes. **C)** M1085T- Memory markers analysis on TIL product from the Gen 2 and Gen 3 processes.

**[00143] Figure 12:** L4054 Activation and exhaustion markers **(A)** Gated on CD4+, **(B)** Gated on CD8+.

**[00144] Figure 13:** L4055 Activation and exhaustion markers **(A)** Gated on CD4+, **(B)** Gated on CD8+.

**[00145] Figure 14:** IFNγ production (pg/mL): (A) L4054, (B) L4055, and (C) M1085T for the Gen 2 and Gen 3 processes: Each bar represented here is mean + SEM for IFNγ levels of stimulated, unstimulated, and media control. Optical density measured at 450 nm.

**[00146] Figure 15:** ELISA analysis of IL-2 concentration in cell culture supernatant: **(A) L4054** and **(B) L4055**. Each bar represented here is mean + SEM for IL-2 levels on spent media. Optical density measured at 450 nm.

**[00147] Figure 16:** Quantification of glucose and lactate (g/L) in spent media: (A) Glucose and (B) Lactate: In the two tumor lines, and in both processes, a decrease in glucose was observed throughout the REP expansion. Conversely, as expected, an increase in lactate was observed. Both the decrease in glucose and the increase in lactate were comparable between the Gen 2 and Gen 3 processes.

**[00148] Figure 17: A)** Quantification of L-glutamine in spent media for L4054 and L4055. **B)** Quantification of Glutamax in spent media for L4054 and L4055. C) Quantification of ammonia in spent media for L4054 and L4055.

**[00149] Figure 18: Telomere length analysis.** The relative telomere length (RTL) value indicates that the average telomere fluorescence per chromosome/genome in Gen 2 and Gen 3 process of the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

**[00150] Figure 19:** Unique CDR3 sequence analysis for TIL final product on L4054 and L4055 under Gen 2 and Gen 3 process. Columns show the number of unique TCR B clonotypes identified from $1 \times 10^6$ cells collected on Harvest Day Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16). Gen 3 shows higher clonal diversity compared to Gen 2 based on the number of unique peptide CDRs within the sample.

**[00151] Figure 20:** Frequency of unique CDR3 sequences on L4054 IL harvested final cell product (Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16)).

**[00152] Figure 21:** Frequency of unique CDR3 sequences on L4055 TIL harvested final cell product (Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16)).

**[00153] Figure 22:** Diversity Index for TIL final product on L4054 and L4055 under Gen 2 and Gen 3 process. Shanon entropy diversity index is a more reliable and common metric for comparison. Gen 3 L4054 and L4055 showed a slightly higher diversity than Gen 2.

**[00154] Figure 23:** Raw data for cell counts Day 7-Gen 3 REP initiation presented in Table 45 (see Example 8 below).

**[00155] Figure 24:** Raw data for cell counts Day 11-Gen 2 REP initiation and Gen 3 Scale Up presented in Table 45 (see Example 8 below).

**[00156] Figure 25:** Raw data for cell counts Day 16-Gen 2 Scale Up and Gen 3 Harvest (*e.g.*, day 16) presented in Table 46 (see Example 8 below).

**[00157] Figure 26:** Raw data for cell counts Day 22-Gen 2 Harvest (*e.g.*, day 22) presented in Table 46 (see Example 8 below). For L4054 Gen 2, post LOVO count was extrapolated to 4 flasks, because was the total number of the study. 1 flask was contaminated, and the extrapolation was done for total = 6.67E+10.

**[00158] Figure 27:** Raw data for flow cytometry results depicted in Figs. 10A, 10A, and 10B.

**[00159] Figure 28:** Raw data for flow cytometry results depicted in Figs. 10C and 10C.

**[00160] Figure 29:** Raw data for flow cytometry results depicted in Figs. 12 and 13.

**[00161] Figure 30:** Raw data for IFNγ production assay results for L4054 samples depicted in Fig. 14.

**[00162] Figure 31:** Raw data for IFNγ production assay results for L4055 samples depicted in Fig. 14.

**[00163] Figure 32:** Raw data for IFNγ production assay results for M1085T samples depicted in Fig. 14.

**[00164] Figure 33:** Raw data for IL-2 ELISA assay results depicted in Fig. 15.

**[00165] Figure 34:** Raw data for the metabolic substrate and metabolic analysis results presented in Figs. 16 and 17.

**[00166] Figure 35:** Raw data for the relative telomere length anaylsis results presented in Fig. 18.

**[00167] Figure 36:** Raw data for the unique CD3 sequence and clonal diversity analyses results presented in Figs. 19 and 22.

**[00168] Figure 37:** Shows a comparison between various Gen 2 (2A process) and the Gen 3.1 process embodiment.

**[00169] Figure 38:** Table describing various features of embodiments of the Gen 2, Gen 2.1 and Gen 3.0 process.

**[00170] Figure 39:** Overview of the media conditions for an embodiment of the Gen 3 process, referred to as Gen 3.1.

**[00171] Figure 40:** Table describing various features of embodiments of the Gen 2, Gen 2.1 and Gen 3.0 process.

**[00172] Figure 41:** Table comparing various features of embodiments of the Gen 2 and Gen 3.0 processes.

**[00173] Figure 42:** Table providing media uses in the various embodiments of the described expansion processes.

**[00174] Figure 43:** Phenotype comparison: Gen 3.0 and Gen 3.1 embodiments of the process showed comparable CD28, CD27 and CD57 expression.

**[00175] Figure 44:** Higher production of IFNγ on Gen 3 final product. IFNγ analysis (by ELISA) was assessed in the culture frozen supernatant to compared both processes. For each tumor overnight stimulation with coated anti -CD3 plate, using fresh TIL product on each Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 16). Each bar represents here are IFNγlevels of stimulated, unstimulated and media control.

**[00176] Figure 45:** Top: Unique CDR3 sequence analysis for TIL final product: Columns show the number of unique TCR B clonotypes identified from $1 \times 10^6$ cells collected on Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16). Gen 3 shows higher clonal diversity compared to Gen 2 based on the number of unique peptide CDRs within the sample. Bottom: Diversity Index for TIL final product: Shanon entropy diversity index is a more reliable a common metric for comparison. Gen 3 showed a slightly higher diversity than Gen 2.

**[00177] Figure 46:** 199 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 97.07% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

**[00178] Figure 47:** 1833 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 99.45% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

**[00179] Figure 48:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

**[00180] Figure 49:** Schematic of an exemplary embodiment of a method for expanding T cells from hematopoietic malignancies using Gen 3 expansion platform.

**[00181] Figure 50:** Provides the structures I-A and I-B, the cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from e.g., 4-1BBL or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second trivalent protein through IgG1-Fc (including CH3 and CH2 domains) is then used to link two of the trivalent proteins together through disulfide bonds (small elongated ovals), stabilizing the structure and providing an agonists capable of bringing together the intracellular signaling domains of the six receptors and signaling proteins to form a signaling complex. The TNFRSF binding domains denoted as cylinders may be scFv domains comprising, e.g., a VH and a VL chain connected by a linker that may comprise hydrophilic residues and Gly and Ser sequences for flexibility, as well as Glu and Lys for solubility.

**[00182] Figure 51:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

**[00183] Figure 52:** Provides a processs overview for an exemplary embodiment (Gen 3.1 Test) of the Gen 3.1 process (a 16 day process).

**[00184] Figure 53:** Provides data from TIL proliferation, average total viable cell counts per tumor fragment, percent viability at Harvest Day and total viable cell counts (TVC) at Harvest Day for exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). Gen 3.1 Test (which includes the addition of OKT-3 and feeders on Day 0) reached maximum capacity of the flask at harvest. If a maximum of 4 flasks are initiated on day 0, each TVC harvest should be multiplied by 4.

**[00185] Figure 54:** Bar graph depicting total viable cell count (TVC) and percent viability for exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test), a 16-day process.

**[00186] Figure 55:** Provides data showing that exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) yielded cells that showed comparable CD28, CD27 and CD57 expression.

**[00187] Figure 56:** Provides data showing TIL memory statuses were comparable across cells yielded by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, and Gen 3.1 Test). Memory statuses of REP TIL are depicted as follows: CD4+ or CD8+ TIL Memory subsets were divided into different memory subsets. Naïve (CD45RA+CD62L+), CM: Central memory (CD45RA-CD62L+), EM: Effector memory (CD45RA-CD62L-), TEMRA/TEFF: RA+ Effector memory/Effectors (CD45RA+CD62L+). Bar graph presented are percentage positive CD45+/-CD62L +/- when gated on CD4+ or CD8+.

**[00188] Figure 57:** Provides data showing TIL activation / exhaustion markers were comparable across cells yielded by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, and Gen 3.1 Test) when gated on CD4+. Activation and exhaustion of REP TIL were determined by multicolor flow cytometry. Harvested TIL samples were stained with flow cytometry antibodies (CD3-BUV395, PD-1-BV421, 2B4/CD244-PB, CD8-BB515, CD25-BUV563, BTLA-PE, KLRG1-PE-Dazzle 594, TIM-3-BV650, CD194/CCR4-APC, CD4-VioGreen, TIGIT-PerCP-eFluor 710, CD183-BV711, CD69-APC-R700, CD95-BUV737, CD127-PE-Cy7, CD103-BV786, LAG-3-APC-eFluor 780). Bar graph presented are percentage of CD4+ or CD8+ TIL of REP TIL.

**[00189] Figure 58:** Provides data showing TIL activation / exhaustion markers were comparable across cells yielded by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.0, Gen 3.1 Control and Gen 3.1) when gated on CD8+. Activation and exhaustion of REP TIL were determined by multicolor flow cytometry. TIL Harvested samples were stained with flow cytometry antibodies (CD3-BUV395, PD-1-BV421, 2B4/CD244-PB, CD8-BB515, CD25-BUV563, BTLA-PE, KLRG1-PE-Dazzle 594, TIM-3-BV650, CD194/CCR4-APC, CD4-VioGreen, TIGIT-PerCP-eFluor 710, CD183-BV711, CD69-APC-R700, CD95-BUV737, CD127-PE-Cy7, CD103-BV786, LAG-3-APC-eFluor 780). Bar graph presented are percentage of CD4+ or CD8+ TIL of REP TIL.

**[00190] Figure 59:** Provides data showing higher production of IFN-γ exhibited by Gen 3.1 final product. IFNγ analysis ELISA was assessed in the culture frozen supernatant to compare both processes. For each tumor overnight stimulation with coated anti -CD3 plate, using fresh TIL product on each Harvest day. Each bar represents here are IFN-γ levels of stimulated, unstimulated and media control.

**[00191] Figure 60:** Provides data showing that IL-2 concentration on supernatant were comparable across exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) using Standard media. Left panel: L4063- Gen 2 Standard Media. Right panel: L4064- CTS Optimizer Media. *ELISA performed with AIM V diluent

**[00192] Figure 61:** Provides data showing that metabolite concentrations were comparable on supernatant supernatants across exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). L4063 TILs were expanded in standard media. L4064 TILs were expanded in CTS Optimizer media.

**[00193] Figure 62:** Telomere length analysis on exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). Telomere length analysis for cells yielded by tumor identification numbers L4063 and L4064: the relative telomere length (RTL) value indicates the average telomere fluorescence per chromosome/genome in cells produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes over the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

**[00194] Figure 63:** Schematic of an exemplary embodiment of the Gen 3.1 Test (Gen 3.1 optimized) process (a 16-17 day process).

**[00195] Figure 64:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

**[00196] Figure 65A-65B:** Comparison tables for exemplary Gen 2 and exemplary Gen 3 processes with exemplary differences highlighted.

**[00197] Figure 66:** Schematic of an exemplary embodiment of the Gen 3 process (a 16/17 day process) preparation timeline.

**[00198] Figure 67:** Schematic of an exemplary embodiment of the Gen 3 process (a 14-16 day process).

**[00199] Figure 68:** Summary of data from Day 16/17 of three engineering runs of an exemplary Gen 3 process embodiment.

**[00200] Figure 69:** Data regarding the extended phenotype of TIL: shown are the differentiation characteristics against TIL identity (ID) specifications for cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

**[00201] Figure 70:** Data regarding the extended phenotype of TIL expanded from lung tumors: shown are the differentiation characteristics against TIL identity (ID) specifications for cells produced by two process development (PD) runs of an exemplary Gen 3 process embodiment using lung tumor tissues.

**[00202] Figure 71:** Data regarding the extended phenotype (purity, identity and memory) of TIL expanded from ovarian tumors: shown are the purity, identity and memory phenotypic characteristics of cells expanded from ovarian tumors using exemplary Gen 2, Gen 3.1, and FR ER (Frozen tumor, Early REP) process embodiments; * indicates condition not tested; Y indicates sampling issue, low TVC count or non-viable cells on thawing.

**[00203] Figure 72:** Shown is the gating strategy for characterization of TIL (gating hierarchy is shown) and data regarding the extended phenotypic characteristics of cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

**[00204] Figure 73:** Shown is the gating strategy for characterization of TIL (gating hierarchy is shown) and data regarding the extended phenotypic characteristics of the CD4+ subpopulation and the CD8+ subpopulation of cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

**[00205] Figure 74:** Shown are data regarding Granzyme B ELISA analysis of cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

**[00206] Figure 75A-75B:** Schematic of an exemplary embodiment of the Gen 3 process (a 16 day process).

**[00207] Figure 76:** Schematic of an exemplary embodiment of the Gen 3 process (a 16 day process).

**[00208] Figure 77:** Comparison of Gen 2, Gen 2.1 and an embodiment of the Gen 3 process (a 16 day process).

**[00209] Figure 78:** Comparison of Gen 2, Gen 2.1 and an embodiment of the Gen 3 process (a 16 day process).

**[00210] Figure 79:** Gen 3 embodiment components.

**[00211] Figure 80:** Gen 3 embodiment flow chart comparison (Gen 3.0, Gen 3.1 control, Gen 3.1 Test).

**[00212] Figure 81:** Total viable cell count and fold expansion are presented for exemplary Gen 3 embodiments (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and serum free cell culture media.

**[00213] Figure 82:** % viability scores upon reactivation, culture scale up and TIL harvest are presented for exemplary Gen 3 embodiments (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and serum free cell culture media.

**[00214] Figure 83:** Presented is phenotypic characterization of final TIL product produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00215] Figure 84:** Presented is memory marker analysis of TIL product produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00216] Figure 85:** Presented are activation and exhaustion markers of TIL produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media followed by CD4+ gated cell sorting.

**[00217] Figure 86:** Presented are activation and exhaustion markers of TIL produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media followed by CD8+ gated cell sorting.

**[00218] Figure 87:** Presented are IFN-γ production (pg/mL) scores for final TIL product produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00219] Figure 88:** Presented is IL-2 concentration (pg/mL) analysis of spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and

L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00220] Figure 89:** Presented is concentration of glucose (g/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00221] Figure 90:** Presented is concentration of lactate (g/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00222] Figure 91:** Presented is concentration of glutamine (mmol/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00223] Figure 92:** Presented is concentration of glutamax (mmol/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

**[00224] Figure 93:** Presented is concentration of ammonia (mmol/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media. Telomere length analysis on exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). Telomere length analysis for cells yielded by tumor identification numbers L4063 and L4064: the relative telomere length (RTL) value indicates the average telomere fluorescence per chromosome/genome in cells produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes over the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

**[00225] Figure 94:** Telomere length analysis on TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media. Telomere length analysis for cells yielded by tumor identification numbers L4063 and L4064: the relative telomere length (RTL) value

indicates the average telomere fluorescence per chromosome/genome in cells produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes over the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

**[00226] Figure 95:** TCR Vβ repertoire summary for TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media. Described is the clonality of TIL for final TIL product yielded by tumor identification numbers L4063 and L4064 produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes as measured by the TCR Vβ repertoire of unique CDR3 sequences.

**[00227] Figure 96:** Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to frequency of unique CDR3 sequences in TIL harvested product from processing of L4063 tumor samples.

**[00228] Figure 97:** Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to percentage shared unique CDR3 sequences in TIL harvested cell product from processing of L4063 tumor samples: 975 sequences are shared between Gen 3.0 and Gen 3.1 Test final product, equivalent to 88% of top 80% of unique CDR3 sequences from Gen 3.0 shared with Gen 3.1 Test final product.

**[00229] Figure 98:** Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to percentage shared unique CDR3 sequences in TIL harvested cell product for from processing of L4064 tumor samples: 2163 sequences are shared between Gen 3.0 and Gen 3.1 Test final product, equivalent to 87% of top 80% of unique CDR3 sequences from Gen 3.0 shared with Gen 3.1 Test final product.

**[00230] Figure 99:** Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to frequency of unique CDR3 sequences in TIL harvested product from processing of L4064 tumor samples.

**[00231] Figure 100:** Shown are the components of an exemplary embodiment of the Gen 3 process (Gen 3-Optimized, a 16-17 day process).

**[00232] Figure 101:** Acceptance criteria table.

**[00233] Figure 102:** Cell counts reactivation Day.

**[00234] Figure 103:** Cell counts Scale Up Day.

**[00235]** **Figure 104:** Cell counts Harvest L4063.

**[00236]** **Figure 105:** Cell counts Harvest L4064.

**[00237]** **Figure 106:** Flow data.

**[00238]** **Figure 107:** Flow data.

**[00239]** **Figure 108:** Flow data.

**[00240]** **Figure 109:** Flow data.

**[00241]** **Figure 110:** IFN-γ production Data Figure 7-L4063.

**[00242]** **Figure 111:** Data IFN-γ production Figure 7-L4064.

**[00243]** **Figure 112:** ELISA analysis of IL-2 concentration data.

**[00244]** **Figure 113:** Metabolic data summary table.

**[00245]** **Figure 114:** Summary data.

**[00246]** **Figure 115:** Summary data.

**[00247]** **Figure 116:** Shannon diversity index.

## BRIEF DESCRIPTION OF THE SEQUENCE LISTING

**[00248]** SEQ ID NO:1 is the amino acid sequence of the heavy chain of muromonab.

**[00249]** SEQ ID NO:2 is the amino acid sequence of the light chain of muromonab.

**[00250]** SEQ ID NO:3 is the amino acid sequence of a recombinant human IL-2 protein.

**[00251]** SEQ ID NO:4 is the amino acid sequence of aldesleukin.

**[00252]** SEQ ID NO:5 is the amino acid sequence of a recombinant human IL-4 protein.

**[00253]** SEQ ID NO:6 is the amino acid sequence of a recombinant human IL-7 protein.

**[00254]** SEQ ID NO:7 is the amino acid sequence of a recombinant human IL-15 protein.

**[00255]** SEQ ID NO:8 is the amino acid sequence of a recombinant human IL-21 protein.

**[00256]** SEQ ID NO:9 is the amino acid sequence of human 4-1BB.

**[00257]** SEQ ID NO:10 is the amino acid sequence of murine 4-1BB.

**[00258]** SEQ ID NO:11 is the heavy chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00259]** SEQ ID NO:12 is the light chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00260]** SEQ ID NO:13 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00261]** SEQ ID NO:14 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00262]** SEQ ID NO:15 is the heavy chain CDRl for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00263]** SEQ ID NO:16 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00264]** SEQ ID NO:17 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00265]** SEQ ID NO:18 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00266]** SEQ ID NO:19 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00267]** SEQ ID NO:20 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

**[00268]** SEQ ID NO:21 is the heavy chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00269]** SEQ ID NO:22 is the light chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00270]** SEQ ID NO:23 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00271]** SEQ ID NO:24 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00272]** SEQ ID NO:25 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00273]** SEQ ID NO:26 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00274]** SEQ ID NO:27 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00275]** SEQ ID NO:28 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00276]** SEQ ID NO:29 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00277]** SEQ ID NO:30 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

**[00278]** SEQ ID NO:31 is an Fc domain for a TNFRSF agonist fusion protein.

**[00279]** SEQ ID NO:32 is a linker for a TNFRSF agonist fusion protein.

**[00280]** SEQ ID NO:33 is a linker for a TNFRSF agonist fusion protein.

**[00281]** SEQ ID NO:34 is a linker for a TNFRSF agonist fusion protein.

**[00282]** SEQ ID NO:35 is a linker for a TNFRSF agonist fusion protein.

**[00283]** SEQ ID NO:36 is a linker for a TNFRSF agonist fusion protein.

**[00284]** SEQ ID NO:37 is a linker for a TNFRSF agonist fusion protein.

**[00285]** SEQ ID NO:38 is a linker for a TNFRSF agonist fusion protein.

**[00286]** SEQ ID NO:39 is a linker for a TNFRSF agonist fusion protein.

**[00287]** SEQ ID NO:40 is a linker for a TNFRSF agonist fusion protein.

**[00288]** SEQ ID NO:41 is a linker for a TNFRSF agonist fusion protein.

**[00289]** SEQ ID NO:42 is an Fc domain for a TNFRSF agonist fusion protein.

**[00290]** SEQ ID NO:43 is a linker for a TNFRSF agonist fusion protein.

**[00291]** SEQ ID NO:44 is a linker for a TNFRSF agonist fusion protein.

**[00292]** SEQ ID NO:45 is a linker for a TNFRSF agonist fusion protein.

**[00293]** SEQ ID NO:46 is a 4-1BB ligand (4-1BBL) amino acid sequence.

**[00294]** SEQ ID NO:47 is a soluble portion of 4-1BBL polypeptide.

**[00295]** SEQ ID NO:48 is a heavy chain variable region (VH) for the 4-1BB agonist antibody 4B4-1-1 version 1.

**[00296]** SEQ ID NO:49 is a light chain variable region (VL) for the 4-1BB agonist antibody 4B4-1-1 version 1.

**[00297]** SEQ ID NO:50 is a heavy chain variable region (VH) for the 4-1BB agonist antibody 4B4-1-1 version 2.

**[00298]** SEQ ID NO:51 is a light chain variable region (VL) for the 4-1BB agonist antibody 4B4-1-1 version 2.

**[00299]** SEQ ID NO:52 is a heavy chain variable region (VH) for the 4-1BB agonist antibody H39E3-2.

**[00300]** SEQ ID NO:53 is a light chain variable region (VL) for the 4-1BB agonist antibody H39E3-2.

**[00301]** SEQ ID NO:54 is the amino acid sequence of human OX40.

**[00302]** SEQ ID NO:55 is the amino acid sequence of murine OX40.

**[00303]** SEQ ID NO:56 is the heavy chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00304]** SEQ ID NO:57 is the light chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00305]** SEQ ID NO:58 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00306]** SEQ ID NO:59 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00307]** SEQ ID NO:60 is the heavy chain CDRl for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00308]** SEQ ID NO:61 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00309]** SEQ ID NO:62 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00310]** SEQ ID NO:63 is the light chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00311]** SEQ ID NO:64 is the light chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00312]** SEQ ID NO:65 is the light chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

**[00313]** SEQ ID NO:66 is the heavy chain for the OX40 agonist monoclonal antibody 11D4.

**[00314]** SEQ ID NO:67 is the light chain for the OX40 agonist monoclonal antibody 11D4.

**[00315]** SEQ ID NO:68 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 11D4.

**[00316]** SEQ ID NO:69 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 11D4.

**[00317]** SEQ ID NO:70 is the heavy chain CDRl for the OX40 agonist monoclonal antibody 11D4.

**[00318]** SEQ ID NO:71 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

**[00319]** SEQ ID NO:72 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

**[00320]** SEQ ID NO:73 is the light chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

**[00321]** SEQ ID NO:74 is the light chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

**[00322]** SEQ ID NO:75 is the light chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

**[00323]** SEQ ID NO:76 is the heavy chain for the OX40 agonist monoclonal antibody 18D8.

**[00324]** SEQ ID NO:77 is the light chain for the OX40 agonist monoclonal antibody 18D8.

**[00325]** SEQ ID NO:78 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 18D8.

**[00326]** SEQ ID NO:79 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 18D8.

**[00327]** SEQ ID NO:80 is the heavy chain CDRl for the OX40 agonist monoclonal antibody 18D8.

**[00328]** SEQ ID NO:81 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

**[00329]** SEQ ID NO:82 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

**[00330]** SEQ ID NO:83 is the light chain CDR1 for the OX40 agonist monoclonal antibody 18D8.

**[00331]** SEQ ID NO:84 is the light chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

**[00332]** SEQ ID NO:85 is the light chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

**[00333]** SEQ ID NO:86 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody Hu119-122.

**[00334]** SEQ ID NO:87 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody Hu119-122.

**[00335]** SEQ ID NO:88 is the heavy chain CDRl for the OX40 agonist monoclonal antibody Hu119-122.

**[00336]** SEQ ID NO:89 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

**[00337]** SEQ ID NO:90 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

**[00338]** SEQ ID NO:91 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.

**[00339]** SEQ ID NO:92 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

**[00340]** SEQ ID NO:93 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

**[00341]** SEQ ID NO:94 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody Hu106-222.

**[00342]** SEQ ID NO:95 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody Hu106-222.

**[00343]** SEQ ID NO:96 is the heavy chain CDRl for the OX40 agonist monoclonal antibody Hu106-222.

**[00344]** SEQ ID NO:97 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

**[00345]** SEQ ID NO:98 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

**[00346]** SEQ ID NO:99 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.

**[00347]** SEQ ID NO:100 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

**[00348]** SEQ ID NO:101 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

**[00349]** SEQ ID NO:102 is an OX40 ligand (OX40L) amino acid sequence.

**[00350]** SEQ ID NO:103 is a soluble portion of OX40L polypeptide.

**[00351]** SEQ ID NO:104 is an alternative soluble portion of OX40L polypeptide.

**[00352]** SEQ ID NO:105 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 008.

**[00353]** SEQ ID NO:106 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 008.

**[00354]** SEQ ID NO:107 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 011.

**[00355]** SEQ ID NO:108 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 011.

**[00356]** SEQ ID NO:109 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 021.

**[00357]** SEQ ID NO:110 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 021.

**[00358]** SEQ ID NO:111 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 023.

**[00359]** SEQ ID NO:112 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 023.

**[00360]** SEQ ID NO:113 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.

**[00361]** SEQ ID NO:114 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

**[00362]** SEQ ID NO:115 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.

**[00363]** SEQ ID NO:116 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

**[00364]** SEQ ID NO:117 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

**[00365]** SEQ ID NO:118 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

**[00366]** SEQ ID NO:119 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

**[00367]** SEQ ID NO:120 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

**[00368]** SEQ ID NO:121 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

**[00369]** SEQ ID NO:122 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

**[00370]** SEQ ID NO:123 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

**[00371]** SEQ ID NO:124 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

**[00372]** SEQ ID NO:125 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.

**[00373]** SEQ ID NO:126 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

**[00374]** SEQ ID NO:127-462 are currently not assigned.

**[00375]** SEQ ID NO:463 is the heavy chain amino acid sequence of the PD-1 inhibitor nivolumab.

**[00376]** SEQ ID NO:464 is the light chain amino acid sequence of the PD-1 inhibitor nivolumab.

**[00377]** SEQ ID NO:465 is the heavy chain variable region ($V_H$) amino acid sequence of the PD-1 inhibitor nivolumab.

**[00378]** SEQ ID NO:466 is the light chain variable region ($V_L$) amino acid sequence of the PD-1 inhibitor nivolumab.

**[00379]** SEQ ID NO:467 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

**[00380]** SEQ ID NO:468 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

**[00381]** SEQ ID NO:469 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

**[00382]** SEQ ID NO:470 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

**[00383]** SEQ ID NO:471 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

**[00384]** SEQ ID NO:472 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

**[00385]** SEQ ID NO:473 is the heavy chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00386]** SEQ ID NO:474 is the light chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00387]** SEQ ID NO:475 is the heavy chain variable region ($V_H$) amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00388]** SEQ ID NO:476 is the light chain variable region ($V_L$) amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00389]** SEQ ID NO:477 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00390]** SEQ ID NO:478 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00391]** SEQ ID NO:479 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00392]** SEQ ID NO:480 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00393]** SEQ ID NO:481 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00394]** SEQ ID NO:482 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

**[00395]** SEQ ID NO:483 is the heavy chain amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00396]** SEQ ID NO:484 is the light chain amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00397]** SEQ ID NO:485 is the heavy chain variable region ($V_H$) amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00398]** SEQ ID NO:486 is the light chain variable region ($V_L$) amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00399]** SEQ ID NO:487 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00400]** SEQ ID NO:488 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00401]** SEQ ID NO:489 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00402]** SEQ ID NO:490 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00403]** SEQ ID NO:491 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00404]** SEQ ID NO:492 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

**[00405]** SEQ ID NO:493 is the heavy chain amino acid sequence of the PD-L1 inhibitor avelumab.

**[00406]** SEQ ID NO:494 is the light chain amino acid sequence of the PD-L1 inhibitor avelumab.

**[00407]** SEQ ID NO:495 is the heavy chain variable region ($V_H$) amino acid sequence of the PD-L1 inhibitor avelumab.

**[00408]** SEQ ID NO:496 is the light chain variable region ($V_L$) amino acid sequence of the PD-L1 inhibitor avelumab.

**[00409]** SEQ ID NO:497 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

**[00410]** SEQ ID NO:498 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

**[00411]** SEQ ID NO:499 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

**[00412]** SEQ ID NO:500 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

**[00413]** SEQ ID NO:501 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

**[00414]** SEQ ID NO:502 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

**[00415]** SEQ ID NO:503 is the heavy chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00416]** SEQ ID NO:504 is the light chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00417]** SEQ ID NO:505 is the heavy chain variable region ($V_H$) amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00418]** SEQ ID NO:506 is the light chain variable region ($V_L$) amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00419]** SEQ ID NO:507 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00420]** SEQ ID NO:508 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00421]** SEQ ID NO:509 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00422]** SEQ ID NO:510 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00423]** SEQ ID NO:511 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00424]** SEQ ID NO:512 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

**[00425]**

## DETAILED DESCRIPTION OF THE INVENTION

### I. Introduction

**[00426]** Adoptive cell therapy utilizing TILs cultured *ex vivo* by the Rapid Expansion Protocol (REP) has produced successful adoptive cell therapy following host immunosuppression in patients with cancer such as melanoma. Current infusion acceptance parameters rely on readouts of the composition of TILs (*e.g.*, CD28, CD8, or CD4 positivity) and on the numerical folds of expansion and viability of the REP product.

**[00427]** Current REP protocols give little insight into the health of the TIL that will be infused into the patient. T cells undergo a profound metabolic shift during the course of their maturation from naïve to effector T cells (see Chang, *et al.*, *Nat. Immunol.* **2016,** *17,* 364, hereby expressly incorporated in its entirety, and in particular for the discussion and markers of anaerobic and aerobic metabolism). For example, naïve T cells rely on mitochondrial respiration to produce ATP, while mature, healthy effector T cells such as TIL are highly glycolytic, relying on aerobic glycolysis to provide the bioenergetics substrates they require for proliferation, migration, activation, and anti-tumor efficacy.

**[00428]** Current TIL manufacturing and treatment processes are limited by length, cost, sterility concerns, and other factors described herein such that the potential to treat patients which are refractory to anti-PD1 and as such have been severly limited. There is an urgent need to provide TIL manufacturing processes and therapies based on such processes that are appropriate for use in treating patients for whom very few or no viable treatment options remain. The present invention meets this need by providing a shortened manufacturing process for use in generating TILs which can then be employed in the treatment of non-small cell lung carcinoma (NSCLC) patients whom are refractory to anti-PD-1 treatment.

### II. Definitions

**[00429]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference in their entireties.

**[00430]** The terms “co-administration,” “co-administering,” “administered in combination with,” “administering in combination with,” “simultaneous,” and “concurrent,”

as used herein, encompass administration of two or more active pharmaceutical ingredients (in a preferred embodiment of the present invention, for example, a plurality of TILs) to a subject so that both active pharmaceutical ingredients and/or their metabolites are present in the subject at the same time. Co-administration includes simultaneous administration in separate compositions, administration at different times in separate compositions, or administration in a composition in which two or more active pharmaceutical ingredients are present. Simultaneous administration in separate compositions and administration in a composition in which both agents are present are preferred.

**[00431]** The term "*in vivo*" refers to an event that takes place in a subject's body.

**[00432]** The term "*in vitro*" refers to an event that takes places outside of a subject's body. In vitro assays encompass cell-based assays in which cells alive or dead are employed and may also encompass a cell-free assay in which no intact cells are employed.

**[00433]** The term "*ex vivo*" refers to an event which involves treating or performing a procedure on a cell, tissue and/or organ which has been removed from a subject's body. Aptly, the cell, tissue and/or organ may be returned to the subject's body in a method of surgery or treatment.

**[00434]** The term "rapid expansion" means an increase in the number of antigen-specific TILs of at least about 3-fold (or 4-, 5-, 6-, 7-, 8-, or 9-fold) over a period of a week, more preferably at least about 10-fold (or 20-, 30-, 40-, 50-, 60-, 70-, 80-, or 90-fold) over a period of a week, or most preferably at least about 100-fold over a period of a week. A number of rapid expansion protocols are described herein.

**[00435]**

**[00436]** By "tumor infiltrating lymphocytes" or "TILs" herein is meant a population of cells originally obtained as white blood cells that have left the bloodstream of a subject and migrated into a tumor. TILs include, but are not limited to, $CD8^+$ cytotoxic T cells (lymphocytes), Th1 and Th17 $CD4^+$ T cells, natural killer cells, dendritic cells and M1 macrophages. TILs include both primary and secondary TILs. "Primary TILs" are those that are obtained from patient tissue samples as outlined herein (sometimes referred to as "freshly harvested"), and "secondary TILs" are any TIL cell populations that have been expanded or proliferated as discussed herein, including, but not limited to bulk TILs and expanded TILs ("REP TILs" or "post-REP TILs"). TIL cell populations can include genetically modified TILs.

**[00437]** By "population of cells" (including TILs) herein is meant a number of cells that share common traits. In general, populations generally range from 1 X $10^6$ to 1 X $10^{10}$ in number, with different TIL populations comprising different numbers. For example, initial growth of primary TILs in the presence of IL-2 results in a population of bulk TILs of roughly $1 \times 10^8$ cells. REP expansion is generally done to provide populations of $1.5 \times 10^9$ to $1.5 \times 10^{10}$ cells for infusion.

**[00438]** By "cryopreserved TILs" herein is meant that TILs, either primary, bulk, or expanded (REP TILs), are treated and stored in the range of about -150°C to -60°C. General methods for cryopreservation are also described elsewhere herein, including in the Examples. For clarity, "cryopreserved TILs" are distinguishable from frozen tissue samples which may be used as a source of primary TILs.

**[00439]** By "thawed cryopreserved TILs" herein is meant a population of TILs that was previously cryopreserved and then treated to return to room temperature or higher, including but not limited to cell culture temperatures or temperatures wherein TILs may be administered to a patient.

**[00440]** TILs can generally be defined either biochemically, using cell surface markers, or functionally, by their ability to infiltrate tumors and effect treatment. TILs can be generally categorized by expressing one or more of the following biomarkers: CD4, CD8, TCR αβ, CD27, CD28, CD56, CCR7, CD45Ra, CD95, PD-1, and CD25. Additionally and alternatively, TILs can be functionally defined by their ability to infiltrate solid tumors upon reintroduction into a patient.

**[00441]** The term "cryopreservation media" or "cryopreservation medium" refers to any medium that can be used for cryopreservation of cells. Such media can include media comprising 7% to 10% DMSO. Exemplary media include CryoStor CS10, Hyperthermasol, as well as combinations thereof. The term "CS10" refers to a cryopreservation medium which is obtained from Stemcell Technologies or from Biolife Solutions. The CS10 medium may be referred to by the trade name "CryoStor® CS10". The CS10 medium is a serum-free, animal component-free medium which comprises DMSO.

**[00442]** The term "central memory T cell" refers to a subset of T cells that in the human are CD45R0+ and constitutively express CCR7 ($CCR7^{hi}$) and CD62L ($CD62^{hi}$). The surface phenotype of central memory T cells also includes TCR, CD3, CD127 (IL-7R), and IL-15R. Transcription factors for central memory T cells include BCL-6, BCL-6B, MBD2, and BMI1.

Central memory T cells primarily secret IL-2 and CD40L as effector molecules after TCR triggering. Central memory T cells are predominant in the CD4 compartment in blood, and in the human are proportionally enriched in lymph nodes and tonsils.

**[00443]** The term "effector memory T cell" refers to a subset of human or mammalian T cells that, like central memory T cells, are CD45R0+, but have lost the constitutive expression of CCR7 ($CCR7^{lo}$) and are heterogeneous or low for CD62L expression ($CD62L^{lo}$). The surface phenotype of central memory T cells also includes TCR, CD3, CD127 (IL-7R), and IL-15R. Transcription factors for central memory T cells include BLIMP1. Effector memory T cells rapidly secret high levels of inflammatory cytokines following antigenic stimulation, including interferon-γ, IL-4, and IL-5. Effector memory T cells are predominant in the CD8 compartment in blood, and in the human are proportionally enriched in the lung, liver, and gut. CD8+ effector memory T cells carry large amounts of perforin.

**[00444]** The term "closed system" refers to a system that is closed to the outside environment. Any closed system appropriate for cell culture methods can be employed with the methods of the present invention. Closed systems include, for example, but are not limited to closed G-containers. Once a tumor segment is added to the closed system, the system is no opened to the outside environment until the TILs are ready to be administered to the patient.

**[00445]** The terms "fragmenting," "fragment," and "fragmented," as used herein to describe processes for disrupting a tumor, includes mechanical fragmentation methods such as crushing, slicing, dividing, and morcellating tumor tissue as well as any other method for disrupting the physical structure of tumor tissue.

**[00446]** The terms "peripheral blood mononuclear cells" and "PBMCs" refers to a peripheral blood cell having a round nucleus, including lymphocytes (T cells, B cells, NK cells) and monocytes. When used as an antigen presenting cell (PBMCs are a type of antigen-presenting cell), the peripheral blood mononuclear cells are preferably irradiated allogeneic peripheral blood mononuclear cells.

**[00447]** The terms "peripheral blood lymphocytes" and "PBLs" refer to T cells expanded from peripheral blood. In some embodiments, PBLs are separated from whole blood or apheresis product from a donor. In some embodiments, PBLs are separated from whole

blood or apheresis product from a donor by positive or negative selection of a T cell phenotype, such as the T cell phenotype of CD3+ CD45+.

**[00448]** The term "anti-CD3 antibody" refers to an antibody or variant thereof, *e.g.*, a monoclonal antibody and including human, humanized, chimeric or murine antibodies which are directed against the CD3 receptor in the T cell antigen receptor of mature T cells. Anti-CD3 antibodies include OKT-3, also known as muromonab. Anti-CD3 antibodies also include the UHCT1 clone, also known as T3 and CD3ε. Other anti-CD3 antibodies include, for example, otelixizumab, teplizumab, and visilizumab.

**[00449]** The term "OKT-3" (also referred to herein as "OKT3") refers to a monoclonal antibody or biosimilar or variant thereof, including human, humanized, chimeric, or murine antibodies, directed against the CD3 receptor in the T cell antigen receptor of mature T cells, and includes commercially-available forms such as OKT-3 (30 ng/mL, MACS GMP CD3 pure, Miltenyi Biotech, Inc., San Diego, CA, USA) and muromonab or variants, conservative amino acid substitutions, glycoforms, or biosimilars thereof. The amino acid sequences of the heavy and light chains of muromonab are given in Table 1 (SEQ ID NO:1 and SEQ ID NO:2). A hybridoma capable of producing OKT-3 is deposited with the American Type Culture Collection and assigned the ATCC accession number CRL 8001. A hybridoma capable of producing OKT-3 is also deposited with European Collection of Authenticated Cell Cultures (ECACC) and assigned Catalogue No. 86022706.

TABLE 1. Amino acid sequences of muromonab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:1<br>Muromonab heavy chain | QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY<br>NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA<br>KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL<br>YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN<br>STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE<br>LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:2<br>Muromonab light chain | QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH<br>FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS<br>SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL<br>TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC | 60<br>120<br>180<br>213 |

**[00450]** The term "IL-2" (also referred to herein as "IL2") refers to the T cell growth factor known as interleukin-2, and includes all forms of IL-2 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-2 is described, *e.g.*, in Nelson, *J. Immunol.* **2004,** *172,* 3983-88 and Malek, *Annu. Rev. Immunol.* **2008,** *26,* 453-79, the disclosures of which are incorporated by reference herein.

The amino acid sequence of recombinant human IL-2 suitable for use in the invention is given in Table 2 (SEQ ID NO:3). For example, the term IL-2 encompasses human, recombinant forms of IL-2 such as aldesleukin (PROLEUKIN, available commercially from multiple suppliers in 22 million IU per single use vials), as well as the form of recombinant IL-2 commercially supplied by CellGenix, Inc., Portsmouth, NH, USA (CELLGRO GMP) or ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-209-b) and other commercial equivalents from other vendors. Aldesleukin (des-alanyl-1, serine-125 human IL-2) is a nonglycosylated human recombinant form of IL-2 with a molecular weight of approximately 15 kDa. The amino acid sequence of aldesleukin suitable for use in the invention is given in Table 2 (SEQ ID NO:4). The term IL-2 also encompasses pegylated forms of IL-2, as described herein, including the pegylated IL2 prodrug NKTR-214, available from Nektar Therapeutics, South San Francisco, CA, USA. NKTR-214 and pegylated IL-2 suitable for use in the invention is described in U.S. Patent Application Publication No. US 2014/0328791 A1 and International Patent Application Publication No. WO 2012/065086 Al, the disclosures of which are incorporated by reference herein. Alternative forms of conjugated IL-2 suitable for use in the invention are described in U.S. Patent Nos. 4,766,106, 5,206,344, 5,089,261 and 4902,502, the disclosures of which are incorporated by reference herein. Formulations of IL-2 suitable for use in the invention are described in U.S. Patent No. 6,706,289, the disclosure of which is incorporated by reference herein.

TABLE 2. Amino acid sequences of interleukins.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:3<br>recombinant<br>human IL-2<br>(rhIL-2) | MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL<br>EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN<br>RWITFCQSII STLT | 60<br>120<br>134 |
| SEQ ID NO:4<br>Aldesleukin | PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE<br>ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW<br>ITFSQSIIST LT | 60<br>120<br>132 |
| SEQ ID NO:5<br>recombinant<br>human IL-4<br>(rhIL-4) | MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH<br>EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI<br>MREKYSKCSS | 60<br>120<br>130 |
| SEQ ID NO:6<br>recombinant<br>human IL-7<br>(rhIL-7) | MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA<br>ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL<br>KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH | 60<br>120<br>153 |
| SEQ ID NO:7<br>recombinant<br>human IL-15<br>(rhIL-15) | MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI<br>HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS | 60<br>115 |
| SEQ ID NO:8<br>recombinant<br>human IL-21<br>(rhIL-21) | MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG<br>NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ<br>HLSSRTHGSE DS | 60<br>120<br>132 |

**[00451]** The term "IL-4" (also referred to herein as "IL4") refers to the cytokine known as interleukin 4, which is produced by Th2 T cells and by eosinophils, basophils, and mast cells. IL-4 regulates the differentiation of naïve helper T cells (Th0 cells) to Th2 T cells. Steinke and Borish, *Respir. Res.* **2001,** *2,* 66-70. Upon activation by IL-4, Th2 T cells subsequently produce additional IL-4 in a positive feedback loop. IL-4 also stimulates B cell proliferation and class II MHC expression, and induces class switching to IgE and $IgG_1$ expression from B cells. Recombinant human IL-4 suitable for use in the invention is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-211) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15 recombinant protein, Cat. No. Gibco CTP0043). The amino acid sequence of recombinant human IL-4 suitable for use in the invention is given in Table 2 (SEQ ID NO:5).

**[00452]** The term "IL-7" (also referred to herein as "IL7") refers to a glycosylated tissue-derived cytokine known as interleukin 7, which may be obtained from stromal and epithelial cells, as well as from dendritic cells. Fry and Mackall, *Blood* **2002,** *99,* 3892-904. IL-7 can stimulate the development of T cells. IL-7 binds to the IL-7 receptor, a heterodimer consisting of IL-7 receptor alpha and common gamma chain receptor, which in a series of signals important for T cell development within the thymus and survival within the periphery. Recombinant human IL-7 suitable for use in the invention is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-254) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15 recombinant protein, Cat. No. Gibco PHC0071). The amino acid sequence of recombinant human IL-7 suitable for use in the invention is given in Table 2 (SEQ ID NO:6).

**[00453]** The term "IL-15" (also referred to herein as "IL15") refers to the T cell growth factor known as interleukin-15, and includes all forms of IL-2 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-15 is described, *e.g.*, in Fehniger and Caligiuri, *Blood* **2001,** *97,* 14-32, the disclosure of which is incorporated by reference herein. IL-15 shares β and γ signaling receptor subunits with IL-2. Recombinant human IL-15 is a single, non-glycosylated polypeptide chain containing 114 amino acids (and an N-terminal methionine) with a molecular mass of 12.8 kDa. Recombinant human IL-15 is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-230-b) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15

recombinant protein, Cat. No. 34-8159-82). The amino acid sequence of recombinant human IL-15 suitable for use in the invention is given in Table 2 (SEQ ID NO:7).

**[00454]** The term "IL-21" (also referred to herein as "IL21") refers to the pleiotropic cytokine protein known as interleukin-21, and includes all forms of IL-21 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-21 is described, *e.g.*, in Spolski and Leonard, *Nat. Rev. Drug. Disc.* **2014,** *13,* 379-95, the disclosure of which is incorporated by reference herein. IL-21 is primarily produced by natural killer T cells and activated human $CD4^+$ T cells. Recombinant human IL-21 is a single, non-glycosylated polypeptide chain containing 132 amino acids with a molecular mass of 15.4 kDa. Recombinant human IL-21 is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-408-b) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-21 recombinant protein, Cat. No. 14-8219-80). The amino acid sequence of recombinant human IL-21 suitable for use in the invention is given in Table 2 (SEQ ID NO:8).

**[00455]** When "an anti-tumor effective amount", "an tumor-inhibiting effective amount", or "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the tumor infiltrating lymphocytes (e.g. secondary TILs or genetically modified cytotoxic lymphocytes) described herein may be administered at a dosage of $10^4$ to $10^{11}$ cells/kg body weight (e.g., $10^5$ to $10^6$, $10^5$ to $10^{10}$, $10^5$ to $10^{11}$, $10^6$ to $10^{10}$, $10^6$ to $10^{11}$,$10^7$ to $10^{11}$, $10^7$ to $10^{10}$, $10^8$ to $10^{11}$, $10^8$ to $10^{10}$, $10^9$ to $10^{11}$, or $10^9$ to $10^{10}$ cells/kg body weight), including all integer values within those ranges. Tumor infiltrating lymphocytes (including in some cases, genetically modified cytotoxic lymphocytes) compositions may also be administered multiple times at these dosages. The tumor infiltrating lymphocytes (inlcuding in some cases, genetically) can be administered by using infusion techniques that are commonly known in immunotherapy (*see,* e.g., Rosenberg et al., *New Eng. J. of Med.* 319: 1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

**[00456]** The term "hematological malignancy", "hematologic malignancy" or terms of correlative meaning refer to mammalian cancers and tumors of the hematopoietic and

lymphoid tissues, including but not limited to tissues of the blood, bone marrow, lymph nodes, and lymphatic system. Hematological malignancies are also referred to as "liquid tumors." Hematological malignancies include, but are not limited to, acute lymphoblastic leukemia (ALL), chronic lymphocytic lymphoma (CLL), small lymphocytic lymphoma (SLL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute monocytic leukemia (AMoL), Hodgkin's lymphoma, and non-Hodgkin's lymphomas. The term "B cell hematological malignancy" refers to hematological malignancies that affect B cells.

**[00457]** The term "liquid tumor" refers to an abnormal mass of cells that is fluid in nature. Liquid tumor cancers include, but are not limited to, leukemias, myelomas, and lymphomas, as well as other hematological malignancies. TILs obtained from liquid tumors may also be referred to herein as marrow infiltrating lymphocytes (MILs). TILs obtained from liquid tumors, including liquid tumors circulating in peripheral blood, may also be referred to herein as PBLs. The terms MIL, TIL, and PBL are used interchangeably herein and differ only based on the tissue type from which the cells are derived.

**[00458]** The term "microenvironment," as used herein, may refer to the solid or hematological tumor microenvironment as a whole or to an individual subset of cells within the microenvironment. The tumor microenvironment, as used herein, refers to a complex mixture of "cells, soluble factors, signaling molecules, extracellular matrices, and mechanical cues that promote neoplastic transformation, support tumor growth and invasion, protect the tumor from host immunity, foster therapeutic resistance, and provide niches for dominant metastases to thrive," as described in Swartz, *et al., Cancer Res.*, **2012,** *72,* 2473. Although tumors express antigens that should be recognized by T cells, tumor clearance by the immune system is rare because of immune suppression by the microenvironment.

**[00459]** In an embodiment, the invention includes a method of treating a cancer with a population of TILs, wherein a patient is pre-treated with non-myeloablative chemotherapy prior to an infusion of TILs according to the invention. In some embodiments, the population of TILs may be provided wherein a patient is pre-treated with nonmyeloablative chemotherapy prior to an infusion of TILs according to the present invention. In an embodiment, the non-myeloablative chemotherapy is cyclophosphamide 60 mg/kg/d for 2 days (days 27 and 26 prior to TIL infusion) and fludarabine 25 mg/m2/d for 5 days (days 27 to 23 prior to TIL infusion). In an embodiment, after non-myeloablative chemotherapy and

TIL infusion (at day 0) according to the invention, the patient receives an intravenous infusion of IL-2 intravenously at 720,000 IU/kg every 8 hours to physiologic tolerance.

**[00460]** Experimental findings indicate that lymphodepletion prior to adoptive transfer of tumor-specific T lymphocytes plays a key role in enhancing treatment efficacy by eliminating regulatory T cells and competing elements of the immune system ("cytokine sinks"). Accordingly, some embodiments of the invention utilize a lymphodepletion step (sometimes also referred to as "immunosuppressive conditioning") on the patient prior to the introduction of the rTILs of the invention.

**[00461]** The term "effective amount" or "therapeutically effective amount" refers to that amount of a compound or combination of compounds as described herein that is sufficient to effect the intended application including, but not limited to, disease treatment. A therapeutically effective amount may vary depending upon the intended application (in vitro or in vivo), or the subject and disease condition being treated (e.g., the weight, age and gender of the subject), the severity of the disease condition, or the manner of administration. The term also applies to a dose that will induce a particular response in target cells (e.g., the reduction of platelet adhesion and/or cell migration). The specific dose will vary depending on the particular compounds chosen, the dosing regimen to be followed, whether the compound is administered in combination with other compounds, timing of administration, the tissue to which it is administered, and the physical delivery system in which the compound is carried.

**[00462]** The terms "treatment", "treating", "treat", and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment", as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development or progression; and (c) relieving the disease, i.e., causing regression of the disease and/or relieving one or more disease symptoms. "Treatment" is also meant to encompass delivery of an agent in order to provide for a pharmacologic effect, even in the absence of a disease or condition. For example, "treatment" encompasses delivery of a composition that can elicit an immune response or confer immunity in the absence of a disease condition, e.g., in the case of a vaccine.

**[00463]** The term "heterologous" when used with reference to portions of a nucleic acid or protein indicates that the nucleic acid or protein comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source, or coding regions from different sources. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

**[00464]** The terms "sequence identity," "percent identity," and "sequence percent identity" (or synonyms thereof, e.g., "99% identical") in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity can be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software are known in the art that can be used to obtain alignments of amino acid or nucleotide sequences. Suitable programs to determine percent sequence identity include for example the BLAST suite of programs available from the U.S. Government's National Center for Biotechnology Information BLAST web site. Comparisons between two sequences can be carried using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. ALIGN, ALIGN-2 (Genentech, South San Francisco, California) or MegAlign, available from DNASTAR, are additional publicly available software programs that can be used to align sequences. One skilled in the art can determine appropriate parameters for maximal alignment by particular alignment software. In certain embodiments, the default parameters of the alignment software are used.

**[00465]** As used herein, the term "variant" encompasses but is not limited to antibodies or fusion proteins which comprise an amino acid sequence which differs from the amino acid sequence of a reference antibody by way of one or more substitutions, deletions and/or additions at certain positions within or adjacent to the amino acid sequence of the reference antibody. The variant may comprise one or more conservative substitutions in its amino acid sequence as compared to the amino acid sequence of a reference antibody. Conservative substitutions may involve, e.g., the substitution of similarly charged or uncharged amino

acids. The variant retains the ability to specifically bind to the antigen of the reference antibody. The term variant also includes pegylated antibodies or proteins.

**[00466]** By "tumor infiltrating lymphocytes" or "TILs" herein is meant a population of cells originally obtained as white blood cells that have left the bloodstream of a subject and migrated into a tumor. TILs include, but are not limited to, $CD8^+$ cytotoxic T cells (lymphocytes), Th1 and Th17 $CD4^+$ T cells, natural killer cells, dendritic cells and M1 macrophages. TILs include both primary and secondary TILs. "Primary TILs" are those that are obtained from patient tissue samples as outlined herein (sometimes referred to as "freshly harvested"), and "secondary TILs" are any TIL cell populations that have been expanded or proliferated as discussed herein, including, but not limited to bulk TILs, expanded TILs ("REP TILs") as well as "reREP TILs" as discussed herein. reREP TILs can include for example second expansion TILs or second additional expansion TILs (such as, for example, those described in Step D of Figure 8, including TILs referred to as reREP TILs).

**[00467]** TILs can generally be defined either biochemically, using cell surface markers, or functionally, by their ability to infiltrate tumors and effect treatment. TILs can be generally categorized by expressing one or more of the following biomarkers: CD4, CD8, TCR αβ, CD27, CD28, CD56, CCR7, CD45Ra, CD95, PD-1, and CD25. Additionally, and alternatively, TILs can be functionally defined by their ability to infiltrate solid tumors upon reintroduction into a patient. TILS may further be characterized by potency – for example, TILS may be considered potent if, for example, interferon (IFN) release is greater than about 50 pg/mL, greater than about 100 pg/mL, greater than about 150 pg/mL, or greater than about 200 pg/mL.

**[00468]** The term "deoxyribonucleotide" encompasses natural and synthetic, unmodified and modified deoxyribonucleotides. Modifications include changes to the sugar moiety, to the base moiety and/or to the linkages between deoxyribonucleotide in the oligonucleotide.

**[00469]** The term "RNA" defines a molecule comprising at least one ribonucleotide residue. The term "ribonucleotide" defines a nucleotide with a hydroxyl group at the 2' position of a b-D-ribofuranose moiety. The term RNA includes double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Nucleotides of the RNA molecules described herein may also comprise non-standard

nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

**[00470]** The terms "pharmaceutically acceptable carrier" or "pharmaceutically acceptable excipient" are intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and inert ingredients. The use of such pharmaceutically acceptable carriers or pharmaceutically acceptable excipients for active pharmaceutical ingredients is well known in the art. Except insofar as any conventional pharmaceutically acceptable carrier or pharmaceutically acceptable excipient is incompatible with the active pharmaceutical ingredient, its use in therapeutic compositions of the invention is contemplated. Additional active pharmaceutical ingredients, such as other drugs, can also be incorporated into the described compositions and methods.

**[00471]** The terms "about" and "approximately" mean within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, more preferably still within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the terms "about" or "approximately" depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art. Moreover, as used herein, the terms "about" and "approximately" mean that dimensions, sizes, formulations, parameters, shapes and other quantities and characteristics are not and need not be exact, but may be approximate and/or larger or smaller, as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art. In general, a dimension, size, formulation, parameter, shape or other quantity or characteristic is "about" or "approximate" whether or not expressly stated to be such. It is noted that embodiments of very different sizes, shapes and dimensions may employ the described arrangements.

**[00472]** The transitional terms "comprising," "consisting essentially of," and "consisting of," when used in the appended claims, in original and amended form, define the claim scope with respect to what unrecited additional claim elements or steps, if any, are excluded from the scope of the claim(s). The term "comprising" is intended to be inclusive or open-ended and does not exclude any additional, unrecited element, method, step or material. The term "consisting of" excludes any element, step or material other than those specified in the claim

and, in the latter instance, impurities ordinary associated with the specified material(s). The term "consisting essentially of" limits the scope of a claim to the specified elements, steps or material(s) and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. All compositions, methods, and kits described herein that embody the present invention can, in alternate embodiments, be more specifically defined by any of the transitional terms "comprising," "consisting essentially of," and "consisting of."

**[00473]** The terms "antibody" and its plural form "antibodies" refer to whole immunoglobulins and any antigen-binding fragment ("antigen-binding portion") or single chains thereof. An "antibody" further refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen-binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as $V_H$) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as $V_L$) and a light chain constant region. The light chain constant region is comprised of one domain, $C_L$. The $V_H$ and $V_L$ regions of an antibody may be further subdivided into regions of hypervariability, which are referred to as complementarity determining regions (CDR) or hypervariable regions (HVR), and which can be interspersed with regions that are more conserved, termed framework regions (FR). Each $V_H$ and $V_L$ is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen epitope or epitopes. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

**[00474]** The term "antigen" refers to a substance that induces an immune response. In some embodiments, an antigen is a molecule capable of being bound by an antibody or a TCR if presented by major histocompatibility complex (MHC) molecules. The term "antigen", as used herein, also encompasses T cell epitopes. An antigen is additionally capable of being recognized by the immune system. In some embodiments, an antigen is capable of inducing a humoral immune response or a cellular immune response leading to the activation of B lymphocytes and/or T lymphocytes. In some cases, this may require that the antigen contains or is linked to a Th cell epitope. An antigen can also have one or more epitopes (*e.g.*, B- and T-epitopes). In some embodiments, an antigen will preferably react,

typically in a highly specific and selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be induced by other antigens.

**[00475]** The terms "monoclonal antibody," "mAb," "monoclonal antibody composition," or their plural forms refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Monoclonal antibodies specific to certain receptors can be made using knowledge and skill in the art of injecting test subjects with suitable antigen and then isolating hybridomas expressing antibodies having the desired sequence or functional characteristics. DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

**[00476]** The terms "antigen-binding portion" or "antigen-binding fragment" of an antibody (or simply "antibody portion" or "fragment"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the $V_L$, $V_H$, $C_L$ and CH1 domains; (ii) a F(ab′)2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the $V_H$ and CH1 domains; (iv) a Fv fragment consisting of the $V_L$ and $V_H$ domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment (Ward, *et al.*, *Nature,* **1989,** *341,* 544-546), which may consist of a $V_H$ or a $V_L$ domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, $V_L$ and $V_H$, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the $V_L$ and $V_H$ regions pair to form monovalent molecules known as single

chain Fv (scFv); see, *e.g.*, Bird, *et al.*, *Science* **1988,** *242,* 423-426; and Huston, *et al.*, *Proc. Natl. Acad. Sci. USA* **1988,** *85,* 5879-5883). Such scFv antibodies are also intended to be encompassed within the terms "antigen-binding portion" or "antigen-binding fragment" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

**[00477]** The term "human antibody," as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). The term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

**[00478]** The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In an embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

**[00479]** The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (such as a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in*

*vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the $V_H$ and $V_L$ regions of the recombinant antibodies are sequences that, while derived from and related to human germline $V_H$ and $V_L$ sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

**[00480]** As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by the heavy chain constant region genes.

**[00481]** The phrases "an antibody recognizing an antigen" and "an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen."

**[00482]** The term "human antibody derivatives" refers to any modified form of the human antibody, including a conjugate of the antibody and another active pharmaceutical ingredient or antibody. The terms "conjugate," "antibody-drug conjugate", "ADC," or "immunoconjugate" refers to an antibody, or a fragment thereof, conjugated to another therapeutic moiety, which can be conjugated to antibodies described herein using methods available in the art.

**[00483]** The terms "humanized antibody," "humanized antibodies," and "humanized" are intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences. Humanized forms of non-human (for example, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a 15 hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond

to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones, *et al.*, *Nature* **1986,** *321,* 522-525; Riechmann, *et al.*, *Nature* **1988,** *332,* 323-329; and Presta, *Curr. Op. Struct. Biol.* **1992,** *2,* 593-596. The antibodies described herein may also be modified to employ any Fc variant which is known to impart an improvement (*e.g.*, reduction) in effector function and/or FcR binding. The Fc variants may include, for example, any one of the amino acid substitutions disclosed in International Patent Application Publication Nos. WO 1988/07089 A1, WO 1996/14339 A1, WO 1998/05787 A1, WO 1998/23289 A1, WO 1999/51642 A1, WO 99/58572 A1, WO 2000/09560 A2, WO 2000/32767 A1, WO 2000/42072 A2, WO 2002/44215 A2, WO 2002/060919 A2, WO 2003/074569 A2, WO 2004/016750 A2, WO 2004/029207 A2, WO 2004/035752 A2, WO 2004/063351 A2, WO 2004/074455 A2, WO 2004/099249 A2, WO 2005/040217 A2, WO 2005/070963 A1, WO 2005/077981 A2, WO 2005/092925 A2, WO 2005/123780 A2, WO 2006/019447 A1, WO 2006/047350 A2, and WO 2006/085967 A2; and U.S. Patent Nos. 5,648,260; 5,739,277; 5,834,250; 5,869,046; 6,096,871; 6,121,022; 6,194,551; 6,242,195; 6,277,375; 6,528,624; 6,538,124; 6,737,056; 6,821,505; 6,998,253; and 7,083,784; the disclosures of which are incorporated by reference herein.

**[00484]** The term "chimeric antibody" is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

**[00485]** A "diabody" is a small antibody fragment with two antigen-binding sites. The fragments comprises a heavy chain variable domain ($V_H$) connected to a light chain variable domain ($V_L$) in the same polypeptide chain ($V_H$-$V_L$ or $V_L$-$V_H$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, *e.g.*, European Patent No. EP 404,097, International Patent Publication No. WO 93/11161; and Bolliger, *et al.*, *Proc. Natl. Acad. Sci. USA* **1993,** *90,* 6444-6448.

**[00486]** The term "glycosylation" refers to a modified derivative of an antibody. An aglycoslated antibody lacks glycosylation. Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Aglycosylation may increase the affinity of the antibody for antigen, as described in U.S. Patent Nos. 5,714,350 and 6,350,861. Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (alpha (1,6) fucosyltransferase), such that antibodies expressed in the Ms704, Ms705, and Ms709 cell lines lack fucose on their carbohydrates. The Ms704, Ms705, and Ms709 FUT8−/− cell lines were created by the targeted disruption of the FUT8 gene in CHO/DG44 cells using two replacement vectors (see e.g. U.S. Patent Publication No. 2004/0110704 or Yamane-Ohnuki, *et al., Biotechnol. Bioeng.*, **2004,** *87,* 614-622). As another example, European Patent No. EP 1,176,195 describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation by reducing or eliminating the alpha 1,6 bond-related enzyme, and also describes cell lines which have a low enzyme activity for adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity, for example the rat myeloma cell line YB2/0 (ATCC CRL 1662). International Patent Publication WO 03/035835 describes a variant CHO cell line, Lec 13 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, *et al., J. Biol. Chem.* **2002,** *277,* 26733-26740. International Patent Publication WO 99/54342 describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (e.g., beta(1,4)-N-acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the

engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana, *et al.*, *Nat. Biotech.* **1999,** *17*, 176-180). Alternatively, the fucose residues of the antibody may be cleaved off using a fucosidase enzyme. For example, the fucosidase alpha-L-fucosidase removes fucosyl residues from antibodies as described in Tarentino, *et al.*, *Biochem.* **1975,** *14,* 5516-5523.

**[00487]** "Pegylation" refers to a modified antibody, or a fragment thereof, that typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Pegylation may, for example, increase the biological (e.g., serum) half life of the antibody. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term "polyethylene glycol" is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono ($C_1$-$C_{10}$)alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. The antibody to be pegylated may be an aglycosylated antibody. Methods for pegylation are known in the art and can be applied to the antibodies of the invention, as described for example in European Patent Nos. EP 0154316 and EP 0401384 and U.S. Patent No. 5,824,778, the disclosures of each of which are incorporated by reference herein.

**[00488]** The term "biosimilar" means a biological product, including a monoclonal antibody or protein, that is highly similar to a U.S. licensed reference biological product notwithstanding minor differences in clinically inactive components, and for which there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product. Furthermore, a similar biological or "biosimilar" medicine is a biological medicine that is similar to another biological medicine that has already been authorized for use by the European Medicines Agency. The term "biosimilar" is also used synonymously by other national and regional regulatory agencies. Biological products or biological medicines are medicines that are made by or derived from a biological source, such as a bacterium or yeast. They can consist of relatively small molecules such as human insulin or erythropoietin, or complex molecules such as monoclonal antibodies. For example, if the reference IL-2 protein is aldesleukin (PROLEUKIN), a protein approved by drug regulatory authorities with reference to aldesleukin is a "biosimilar to" aldesleukin or is a "biosimilar thereof" of aldesleukin. In Europe, a similar biological or "biosimilar" medicine is a biological medicine that is similar

to another biological medicine that has already been authorized for use by the European Medicines Agency (EMA). The relevant legal basis for similar biological applications in Europe is Article 6 of Regulation (EC) No 726/2004 and Article 10(4) of Directive 2001/83/EC, as amended and therefore in Europe, the biosimilar may be authorized, approved for authorization or subject of an application for authorization under Article 6 of Regulation (EC) No 726/2004 and Article 10(4) of Directive 2001/83/EC. The already authorized original biological medicinal product may be referred to as a "reference medicinal product" in Europe. Some of the requirements for a product to be considered a biosimilar are outlined in the CHMP Guideline on Similar Biological Medicinal Products. In addition, product specific guidelines, including guidelines relating to monoclonal antibody biosimilars, are provided on a product-by-product basis by the EMA and published on its website. A biosimilar as described herein may be similar to the reference medicinal product by way of quality characteristics, biological activity, mechanism of action, safety profiles and/or efficacy. In addition, the biosimilar may be used or be intended for use to treat the same conditions as the reference medicinal product. Thus, a biosimilar as described herein may be deemed to have similar or highly similar quality characteristics to a reference medicinal product. Alternatively, or in addition, a biosimilar as described herein may be deemed to have similar or highly similar biological activity to a reference medicinal product. Alternatively, or in addition, a biosimilar as described herein may be deemed to have a similar or highly similar safety profile to a reference medicinal product. Alternatively, or in addition, a biosimilar as described herein may be deemed to have similar or highly similar efficacy to a reference medicinal product. As described herein, a biosimilar in Europe is compared to a reference medicinal product which has been authorized by the EMA. However, in some instances, the biosimilar may be compared to a biological medicinal product which has been authorized outside the European Economic Area (a non-EEA authorized "comparator") in certain studies. Such studies include for example certain clinical and in vivo non-clinical studies. As used herein, the term "biosimilar" also relates to a biological medicinal product which has been or may be compared to a non-EEA authorized comparator. Certain biosimilars are proteins such as antibodies, antibody fragments (for example, antigen binding portions) and fusion proteins. A protein biosimilar may have an amino acid sequence that has minor modifications in the amino acid structure (including for example deletions, additions, and/or substitutions of amino acids) which do not significantly affect the function of the polypeptide. The biosimilar may comprise an amino acid sequence having a sequence identity of 97% or greater to the amino acid sequence of its reference

medicinal product, e.g., 97%, 98%, 99% or 100%. The biosimilar may comprise one or more post-translational modifications, for example, although not limited to, glycosylation, oxidation, deamidation, and/or truncation which is/are different to the post-translational modifications of the reference medicinal product, provided that the differences do not result in a change in safety and/or efficacy of the medicinal product. The biosimilar may have an identical or different glycosylation pattern to the reference medicinal product. Particularly, although not exclusively, the biosimilar may have a different glycosylation pattern if the differences address or are intended to address safety concerns associated with the reference medicinal product. Additionally, the biosimilar may deviate from the reference medicinal product in for example its strength, pharmaceutical form, formulation, excipients and/or presentation, providing safety and efficacy of the medicinal product is not compromised. The biosimilar may comprise differences in for example pharmacokinetic (PK) and/or pharmacodynamic (PD) profiles as compared to the reference medicinal product but is still deemed sufficiently similar to the reference medicinal product as to be authorized or considered suitable for authorization. In certain circumstances, the biosimilar exhibits different binding characteristics as compared to the reference medicinal product, wherein the different binding characteristics are considered by a Regulatory Authority such as the EMA not to be a barrier for authorization as a similar biological product. The term "biosimilar" is also used synonymously by other national and regional regulatory agencies.

## III. TIL Manufacturing Processes – 2A

**[00489]** An exemplary TIL process known as process 2A containing some of these features is depicted in Figure 2, and some of the advantages of this embodiment of the present invention over process 1C are described in Figures F and G. An embodiment of process 2A is shown Figure 1.

**[00490]** As discussed herein, the present invention can include a step relating to the restimulation of cryopreserved TILs to increase their metabolic activity and thus relative health prior to transplant into a patient, and methods of testing said metabolic health. As generally outlined herein, TILs are generally taken from a patient sample and manipulated to expand their number prior to transplant into a patient. In some embodiments, the TILs may be optionally genetically manipulated as discussed below.

**[00491]** In some embodiments, the TILs may be cryopreserved. Once thawed, they may also be restimulated to increase their metabolism prior to infusion into a patient.

**[00492]** In some embodiments, the first expansion (including processes referred to as the preREP as well as processes shown in Figure 1 as Step A) is shortened to 3 to 14 days and the second expansion (including processes referred to as the REP as well as processes shown in Figure 1 as Step B) is shorted to 7 to 14 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the first expansion (for example, an expansion described as Step B in Figure 1) is shortened to 11 days and the second expansion (for example, an expansion as described in Step D in Figure 1) is shortened to 11 days. In some embodiments, the combination of the first expansion and second expansion (for example, expansions described as Step B and Step D in Figure 1) is shortened to 22 days, as discussed in detail below and in the examples and figures.

**[00493]** The "Step" Designations A, B, C, *etc.*, below are in reference to Figure 1 and in reference to certain embodiments described herein. The ordering of the Steps below and in Figure 1 is exemplary and any combination or order of steps, as well as additional steps, repetition of steps, and/or omission of steps is contemplated by the present application and the methods disclosed herein.

### A. STEP A: Obtain Patient tumor sample

**[00494]** In general, TILs are initially obtained from a patient tumor sample ("primary TILs") and then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, restimulated as outlined herein and optionally evaluated for phenotype and metabolic parameters as an indication of TIL health.

**[00495]** A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In some embodiments, multilesional sampling is used. In some embodiments, surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells includes multilesional sampling (*i.e.*, obtaining samples from one or more tumor cites and/or locations in the patient, as well as one or more tumors in the same location or in close proximity). In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of lung

tissue. In some embodiments, useful TILs are obtained from non-small cell lung carcinoma (NSCLC).

**[00496]** Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 $mm^3$, with from about 2-3 $mm^3$ being particularly useful. The TILs are cultured from these fragments using enzymatic tumor digests. Such tumor digests may be produced by incubation in enzymatic media (*e.g.*, Roswell Park Memorial Institute (RPMI) 1640 buffer, 2 mM glutamate, 10 mcg/mL gentamicine, 30 units/mL of DNase and 1.0 mg/mL of collagenase) followed by mechanical dissociation (*e.g.*, using a tissue dissociator). Tumor digests may be produced by placing the tumor in enzymatic media and mechanically dissociating the tumor for approximately 1 minute, followed by incubation for 30 minutes at 37 °C in 5% $CO_2$, followed by repeated cycles of mechanical dissociation and incubation under the foregoing conditions until only small tissue pieces are present. At the end of this process, if the cell suspension contains a large number of red blood cells or dead cells, a density gradient separation using FICOLL branched hydrophilic polysaccharide may be performed to remove these cells. Alternative methods known in the art may be used, such as those described in U.S. Patent Application Publication No. 2012/0244133 A1, the disclosure of which is incorporated by reference herein. Any of the foregoing methods may be used in any of the embodiments described herein for methods of expanding TILs or methods treating a cancer.

**[00497]** As indicated above, in some embodiments, the TILs are derived from solid tumors. In some embodiments, the solid tumors are not fragmented. In some embodiments, the solid tumors are not fragmented and are subjected to enzymatic digestion as whole tumors. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% $CO_2$. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% $CO_2$ with rotation. In some embodiments, the tumors are digested overnight with constant rotation. In some embodiments, the tumors are digested overnight at 37°C, 5% $CO_2$ with constant rotation. In some embodiments, the whole tumor is combined with with the enzymes to form a tumor digest reaction mixture.

**[00498]** In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

**[00499]** In some embodiments, the enxyme mixture comprises collagenase. In some embodiments, the collagenase is collagenase IV. In some embodiments, the working stock for the collagenase is a 100 mg/ml 10X working stock.

**[00500]** In some embodiments, the enzyme mixture comprises DNAse. In some embodiments, the working stock for the DNAse is a 10,000IU/ml 10X working stock.

**[00501]** In some embodiments, the enzyme mixture comprises hyaluronidase. In some embodiments, the working stock for the hyaluronidase is a 10-mg/ml 10X working stock.

**[00502]** In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml DNAse, and 1 mg/ml hyaluronidase.

**[00503]** In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml DNAse, and 1 mg/ml hyaluronidase.

**[00504]** In general, the harvested cell suspension is called a "primary cell population" or a "freshly harvested" cell population.

**[00505]** In some embodiments, fragmentation includes physical fragmentation, including for example, dissection as well as digestion. In some embodiments, the fragmentation is physical fragmentation. In some embodiments, the fragmentation is dissection. In some embodiments, the fragmentation is by digestion. In some embodiments, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients. In an embodiment, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients.

**[00506]** In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained in, for example, Step A (as provided in Figure 1). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 40

fragments or pieces are placed in each container for the first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 $mm^3$. In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 $mm^3$ to about 1500 $mm^3$. In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 $mm^3$. In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams. In some embodiments, the multiple fragments comprise about 4 fragments.

**[00507]** In some embodiments, the TILs are obtained from tumor fragments. In some embodiments, the tumor fragment is obtained by sharp dissection. In some embodiments, the tumor fragment is between about 1 $mm^3$ and 10 $mm^3$. In some embodiments, the tumor fragment is between about 1 $mm^3$ and 8 $mm^3$. In some embodiments, the tumor fragment is about 1 $mm^3$. In some embodiments, the tumor fragment is about 2 $mm^3$. In some embodiments, the tumor fragment is about 3 $mm^3$. In some embodiments, the tumor fragment is about 4 $mm^3$. In some embodiments, the tumor fragment is about 5 $mm^3$. In some embodiments, the tumor fragment is about 6 $mm^3$. In some embodiments, the tumor fragment is about 7 $mm^3$. In some embodiments, the tumor fragment is about 8 $mm^3$. In some embodiments, the tumor fragment is about 9 $mm^3$. In some embodiments, the tumor fragment is about 10 $mm^3$. In some embodiments, the tumors are 1-4 mm x 1-4 mm x 1-4 mm. In some embodiments, the tumors are 1 mm x 1 mm x 1 mm. In some embodiments, the tumors are 2 mm x 2 mm x 2 mm. In some embodiments, the tumors are 3 mm x 3 mm x 3 mm. In some embodiments, the tumors are 4 mm x 4 mm x 4 mm.

**[00508]** In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of fatty tissue on each piece.

**[00509]** In some embodiments, the tumor fragmentation is performed in order to maintain the tumor internal structure. In some embodiments, the tumor fragmentation is performed without preforming a sawing motion with a scalpel. In some embodiments, the TILs are obtained from tumor digests. In some embodiments, tumor digests were generated by incubation in enzyme media, for example but not limited to RPMI 1640, 2 mM GlutaMAX,

10 mg/mL gentamicin, 30 U/mL DNase, and 1.0 mg/mL collagenase, followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). After placing the tumor in enzyme media, the tumor can be mechanically dissociated for approximately 1 minute. The solution can then be incubated for 30 minutes at 37 °C in 5% $CO_2$ and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% $CO_2$, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% $CO_2$. In some embodiments, at the end of the final incubation if the cell suspension contained a large number of red blood cells or dead cells, a density gradient separation using Ficoll can be performed to remove these cells.

**[00510]** In some embodiments, the harvested cell suspension prior to the first expansion step is called a "primary cell population" or a "freshly harvested" cell population.

**[00511]** In some embodiments, cells can be optionally frozen after sample harvest and stored frozen prior to entry into the expansion described in Step B, which is described in further detail below, as well as exemplified in Figure 1.

### B. STEP B: First Expansion

**[00512]** In some embodiments, the present methods provide for obtaining young TILs, which are capable of increased replication cycles upon administration to a subject/patient and as such may provide additional therapeutic benefits over older TILs (*i.e.*, TILs which have further undergone more rounds of replication prior to administration to a subject/patient). Features of young TILs have been described in the literature, for example Donia, at al., *Scandinavian Journal of Immunology,* 75:157–167 (2012); Dudley et al., *Clin Cancer Res,* 16:6122-6131 (2010); Huang et al., *J Immunother*, 28(3):258–267 (2005); Besser et al., *Clin Cancer Res*, 19(17):OF1-OF9 (2013); Besser et al., *J Immunother* 32:415–423 (2009); Robbins, et al., *J Immunol* 2004; 173:7125-7130; Shen et al., J Immunother, 30:123–129 (2007); Zhou, et al., *J Immunother*, 28:53–62 (2005); and Tran, et al., J Immunother, 31:742–751 (2008), all of which are incorporated herein by reference in their entireties.

**[00513]** The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding

specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using methods referred to as process 1C, as exemplified in Figure 5 and/or Figure 6. In some embodiments, the TILs obtained in the first expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCRα/β).

**[00514]** After dissection or digestion of tumor fragments, for example such as described in Step A of Figure 1, the resulting cells are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 3 to 14 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 7 to 14 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 10 to 14 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this

primary cell population is cultured for a period of about 11 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells.

**[00515]** In a preferred embodiment, expansion of TILs may be performed using an initial bulk TIL expansion step (for example such as those described in Step B of Figure 1, which can include processes referred to as pre-REP) as described below and herein, followed by a second expansion (Step D, including processes referred to as rapid expansion protocol (REP) steps) as described below under Step D and herein, followed by optional cryopreservation, and followed by a second Step D (including processes referred to as restimulation REP steps) as described below and herein. The TILs obtained from this process may be optionally characterized for phenotypic characteristics and metabolic parameters as described herein.

**[00516]** In embodiments where TIL cultures are initiated in 24-well plates, for example, using Costar 24-well cell culture cluster, flat bottom (Corning Incorporated, Corning, NY, each well can be seeded with $1 \times 10^6$ tumor digest cells or one tumor fragment in 2 mL of complete medium (CM) with IL-2 (6000 IU/mL; Chiron Corp., Emeryville, CA). In some embodiments, the tumor fragment is between about 1 $mm^3$ and 10 $mm^3$.

**[00517]** In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, CM for Step B consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10 $cm^2$ gas-permeable silicon bottom (for example, G-Rex10; Wilson Wolf Manufacturing, New Brighton, MN) (Fig. 1), each flask was loaded with $10–40 \times 10^6$ viable tumor digest cells or 5–30 tumor fragments in 10–40 mL of CM with IL-2. Both the G-Rex10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% $CO_2$ and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days.

**[00518]** After preparation of the tumor fragments, the resulting cells (*i.e.*, fragments) are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum (or, in some cases, as outlined herein, in the presence of aAPC cell population) with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 10 to 14 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, the growth media

during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the IL is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of 20-30×$10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 20×$10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 25×$10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 30×$10^6$ IU/mg for a 1 mg vial. In some embodiments, the IL- 2 stock solution has a final concentration of 4-8×$10^6$ IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of 5-7×$10^6$ IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of 6×$10^6$ IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepare as described in Example 5. In some embodiments, the first expansion culture media comprises about 10,000 IU/mL of IL-2, about 9,000 IU/mL of IL-2, about 8,000 IU/mL of IL-2, about 7,000 IU/mL of IL-2, about 6000 IU/mL of IL-2 or about 5,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 9,000 IU/mL of IL-2 to about 5,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 8,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 7,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 6,000 IU/mL of IL-2. In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium further comprises IL-2. In a preferred embodiment, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or about 8000 IU/mL of IL-2.

**[00519]** In some embodiments, first expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the first expansion

culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the cell culture medium further comprises IL-15. In a preferred embodiment, the cell culture medium comprises about 180 IU/mL of IL-15.

**[00520]** In some embodiments, first expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the cell culture medium comprises about 1 IU/mL of IL-21.

**[00521]** In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 μg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and

50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

TABLE 3: Amino acid sequences of muromonab (exemplary OKT-3 antibody)

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:1<br>Muromonab heavy chain | QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY 60<br>NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA 120<br>KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL 180<br>YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG 240<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN 300<br>STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE 360<br>LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW 420<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK 450 |
| SEQ ID NO:2<br>Muromonab light chain | QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH 60<br>FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS 120<br>SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL 180<br>TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC 213 |

**[00522]** In some embodiments, the cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 μg/mL and 100 μg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 μg/mL and 40 μg/mL.

**[00523]** In some embodiments, in addition to one or more TNFRSF agonists, the cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

**[00524]** In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, it is referred to as CM1 (culture medium 1). In some embodiments, CM consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10cm$^2$ gas-permeable silicon bottom (for example, G-Rex10; Wilson Wolf Manufacturing, New Brighton, MN) (Fig. 1), each flask was loaded with 10–40x10$^6$ viable tumor digest cells

or 5–30 tumor fragments in 10–40mL of CM with IL-2. Both the G-Rex10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% $CO_2$ and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days. In some embodiments, the CM is the CM1 described in the Examples, *see*, Example 1. In some embodiments, the first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the initial cell culture medium or the first cell culture medium comprises IL-2.

**[00525]** In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1, which can include those sometimes referred to as the pre-REP) process is shortened to 3-14 days, as discussed in the examples and figures. In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1, which can include those sometimes referred to as the pre-REP) is shortened to 7 to 14 days, as discussed in the Examples and shown in Figures 4 and 5, as well as including for example, an expansion as described in Step B of Figure 1. In some embodiments, the first expansion of Step B is shortened to 10-14 days. In some embodiments, the first expansion is shortened to 11 days, as discussed in, for example, an expansion as described in Step B of Figure 1.

**[00526]** In some embodiments, the first TIL expansion can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the first TIL expansion can proceed for 1 day to 14 days. In some embodiments, the first TIL expansion can proceed for 2 days to 14 days. In some embodiments, the first TIL expansion can proceed for 3 days to 14 days. In some embodiments, the first TIL expansion can proceed for 4 days to 14 days. In some embodiments, the first TIL expansion can proceed for 5 days to 14 days. In some embodiments, the first TIL expansion can proceed for 6 days to 14 days. In some embodiments, the first TIL expansion can proceed for 7 days to 14 days. In some embodiments, the first TIL expansion can proceed for 8 days to 14 days. In some embodiments, the first TIL expansion can proceed for 9 days to 14 days. In some embodiments, the first TIL expansion can proceed for 10 days to 14 days. In some embodiments, the first TIL expansion can proceed for 11 days to 14 days. In some embodiments, the first TIL expansion can proceed for 12 days to 14 days. In some embodiments, the first TIL expansion can proceed for 13 days to 14 days. In some embodiments, the first TIL expansion can proceed for 14 days. In some embodiments, the

first TIL expansion can proceed for 1 day to 11 days. In some embodiments, the first TIL expansion can proceed for 2 days to 11 days. In some embodiments, the first TIL expansion can proceed for 3 days to 11 days. In some embodiments, the first TIL expansion can proceed for 4 days to 11 days. In some embodiments, the first TIL expansion can proceed for 5 days to 11 days. In some embodiments, the first TIL expansion can proceed for 6 days to 11 days. In some embodiments, the first TIL expansion can proceed for 7 days to 11 days. In some embodiments, the first TIL expansion can proceed for 8 days to 11 days. In some embodiments, the first TIL expansion can proceed for 9 days to 11 days. In some embodiments, the first TIL expansion can proceed for 10 days to 11 days. In some embodiments, the first TIL expansion can proceed for 11 days.

**[00527]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first expansion, including for example during a Step B processes according to Figure 1, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 1 and as described herein.

**[00528]** In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 1) process is shortened to 3 to 14 days, as discussed in the examples and figures. In some embodiments, the first expansion of Step B is shortened to 7 to 14 days. In some embodiments, the first expansion of Step B is shortened to 10 to 14 days. In some embodiments, the first expansion is shortened to 11 days.

**[00529]** In some embodiments, the first expansion, for example, Step B according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

### C. STEP C: First Expansion to Second Expansion Transition

**[00530]** In some cases, the bulk TIL population obtained from the first expansion, including for example the TIL population obtained from for example, Step B as indicated in Figure 1,

can be cryopreserved immediately, using the protocols discussed herein below. Alternatively, the TIL population obtained from the first expansion, referred to as the second TIL population, can be subjected to a second expansion (which can include expansions sometimes referred to as REP) and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the first TIL population (sometimes referred to as the bulk TIL population) or the second TIL population (which can in some embodiments include populations referred to as the REP TIL populations) can be subjected to genetic modifications for suitable treatments prior to expansion or after the first expansion and prior to the second expansion.

**[00531]** In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1) are not stored and proceed directly to the second expansion. In some embodiments, the TILs obtained from the first expansion are not cryopreserved after the first expansion and prior to the second expansion. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 7 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 14 days from when fragmentation occurs.

**[00532]** In some embodiments, the transition from the first expansion to the second expansion occurs at 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 1 day to 14 days from when fragmentation occurs. In some embodiments, the first TIL expansion can proceed for 2 days to 14 days. In some embodiments, the transition from the first expansion to the second expansion occurs 3 days to 14 days from when fragmentation occurs. In some

embodiments, the transition from the first expansion to the second expansion occurs 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 5 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 6 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 7 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 8 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 9 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 10 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 11 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 12 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 13 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 1 day to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 2 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 3 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 4 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 5 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 6 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 7 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 8 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 9 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 10 days

to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 11 days from when fragmentation occurs.

**[00533]** In some embodiments, the TILs are not stored after the first expansion and prior to the second expansion, and the TILs proceed directly to the second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 1). In some embodiments, the transition occurs in closed system, as described herein. In some embodiments, the TILs from the first expansion, the second population of TILs, proceeds directly into the second expansion with no transition period.

**[00534]** In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

1. Cytokines

**[00535]** The expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

**[00536]** Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILS is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in International Publication No. WO 2015/189356 and W International Publication No. WO 2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21 and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

TABLE 4: Amino acid sequences of interleukins.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:3 recombinant human IL-2 (rhIL-2) | MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL<br>EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN<br>RWITFCQSII STLT | 60<br>120<br>134 |
| SEQ ID NO:4 Aldesleukin | PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE<br>ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW<br>ITFSQSIIST LT | 60<br>120<br>132 |
| SEQ ID NO:5 | MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH<br>EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI | 60<br>120 |

| | | |
|---|---|---|
| recombinant human IL-4 (rhIL-4) | MREKYSKCSS | 130 |
| SEQ ID NO:6 recombinant human IL-7 (rhIL-7) | MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA<br>ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL<br>KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH | 60<br>120<br>153 |
| SEQ ID NO:7 recombinant human IL-15 (rhIL-15) | MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI<br>HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS | 60<br>115 |
| SEQ ID NO:8 recombinant human IL-21 (rhIL-21) | MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG<br>NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ<br>HLSSRTHGSE DS | 60<br>120<br>132 |

### D. STEP D: Second Expansion

**[00537]** In some embodiments, the TIL cell population is expanded in number after harvest and initial bulk processing for example, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 1). This further expansion is referred to herein as the second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (REP; as well as processes as indicated in Step D of Figure 1). The second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container.

**[00538]** In some embodiments, the second expansion or second TIL expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 1) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the second TIL expansion can proceed for about 7 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 8 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 9 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 10 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 11 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 12 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 13 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 14 days.

**[00539]** In an embodiment, the second expansion can be performed in a gas permeable container using the methods of the present disclosure (including for example, expansions referred to as REP; as well as processes as indicated in Step D of Figure 1). For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San Diego, CA, USA). TILs can be expanded to induce further stimulation of the TILs *in vitro* by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 μM MART-1 :26-35 (27 L) or gpl 00:209-217 (210M), optionally in the presence of a T-cell growth factor, such as 300 IU/mL IL-2 or IL-15. Other suitable antigens may include, *e.g.*, NY-ESO-1, TRP-1, TRP-2, tyrosinase cancer antigen, MAGE-A3, SSX-2, and VEGFR2, or antigenic portions thereof. TIL may also be rapidly expanded by re-stimulation with the same antigen(s) of the cancer pulsed onto HLA-A2-expressing antigen-presenting cells. Alternatively, the TILs can be further re-stimulated with, *e.g.*, example, irradiated, autologous lymphocytes or with irradiated HLA-A2+ allogeneic lymphocytes and IL-2. In some embodiments, the re-stimulation occurs as part of the second expansion. In some embodiments, the second expansion occurs in the presence of irradiated, autologous lymphocytes or with irradiated HLA-A2+ allogeneic lymphocytes and IL-2.

**[00540]** In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or between 8000 IU/mL of IL-2.

**[00541]** In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 µg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

**[00542]** In some embodiments, the cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 µg/mL and 100 µg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 µg/mL and 40 µg/mL.

**[00543]** In some embodiments, in addition to one or more TNFRSF agonists, the cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

**[00544]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the second expansion, including for example during a Step D processes according to Figure 1, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-15,

and IL-21 as well as any combinations thereof can be included during Step D processes according to Figure 1 and as described herein.

**[00545]** In some embodiments, the second expansion can be conducted in a supplemented cell culture medium comprising IL-2, OKT-3, antigen-presenting feeder cells, and optionally a TNFRSF agonist. In some embodiments, the second expansion occurs in a supplemented cell culture medium. In some embodiments, the supplemented cell culture medium comprises IL-2, OKT-3, and antigen-presenting feeder cells. In some embodiments, the second cell culture medium comprises IL-2, OKT-3, and antigen-presenting cells (APCs; also referred to as antigen-presenting feeder cells). In some embodiments, the second expansion occurs in a cell culture medium comprising IL-2, OKT-3, and antigen-presenting feeder cells (*i.e.*, antigen presenting cells).

**[00546]** In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the cell culture medium further comprises IL-15. In a preferred embodiment, the cell culture medium comprises about 180 IU/mL of IL-15.

**[00547]** In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media

comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the cell culture medium comprises about 1 IU/mL of IL-21.

**[00548]** In some embodiments the antigen-presenting feeder cells (APCs) are PBMCs. In an embodiment, the ratio of TILs to PBMCs and/or antigen-presenting cells in the rapid expansion and/or the second expansion is about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 100 and 1 to 200.

**[00549]** In an embodiment, REP and/or the second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. Media replacement is done (generally 2/3 media replacement via respiration with fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[00550]** In some embodiments, the second expansion (which can include processes referred to as the REP process) is shortened to 7-14 days, as discussed in the examples and figures. In some embodiments, the second expansion is shortened to 11 days.

**[00551]** In an embodiment, REP and/or the second expansion may be performed using T-175 flasks and gas permeable bags as previously described (Tran, *et al.*, *J. Immunother.* **2008,** *31,* 742-51; Dudley, *et al.*, *J. Immunother.* **2003,** *26,* 332-42) or gas permeable cultureware (G-Rex flasks). In some embodiments, the second expansion (including expansions referred to as rapid expansions) is performed in T-175 flasks, and about 1 x $10^6$ TILs suspended in 150 mL of media may be added to each T-175 flask. The TILs may be cultured in a 1 to 1 mixture of CM and AIM-V medium, supplemented with 3000 IU per mL of IL-2 and 30 ng per ml of anti-CD3. The T-175 flasks may be incubated at 37° C in 5% $CO_2$. Half the media may be exchanged on day 5 using 50/50 medium with 3000 IU per mL of IL-2. In some

embodiments, on day 7 cells from two T-175 flasks may be combined in a 3 L bag and 300 mL of AIM V with 5% human AB serum and 3000 IU per mL of IL-2 was added to the 300 ml of TIL suspension. The number of cells in each bag was counted every day or two and fresh media was added to keep the cell count between 0.5 and 2.0 x $10^6$ cells/mL.

**[00552]** In an embodiment, the second expansion (which can include expansions referred to as REP, as well as those referred to in Step D of Figure 1) may be performed in 500 mL capacity gas permeable flasks with 100 cm gas-permeable silicon bottoms (G-Rex 100, commercially available from Wilson Wolf Manufacturing Corporation, New Brighton, MN, USA), 5 × $10^6$ or 10 × $10^6$ TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per ml of anti-CD3 (OKT3). The G-Rex 100 flasks may be incubated at 37°C in 5% $CO_2$. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and centrifuged at 1500 rpm (491 × g) for 10 minutes. The TIL pellets may be re-suspended with 150 mL of fresh medium with 5% human AB serum, 3000 IU per mL of IL-2, and added back to the original G-Rex 100 flasks. When TIL are expanded serially in G-Rex 100 flasks, on day 7 the TIL in each G-Rex 100 may be suspended in the 300 mL of media present in each flask and the cell suspension may be divided into 3 100 mL aliquots that may be used to seed 3 G-Rex 100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU per mL of IL-2 may be added to each flask. The G-Rex 100 flasks may be incubated at 37° C in 5% $CO_2$ and after 4 days 150 mL of AIM-V with 3000 IU per mL of IL-2 may be added to each G-REX 100 flask. The cells may be harvested on day 14 of culture.

**[00553]** In an embodiment, the second expansion (including expansions referred to as REP) is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. In some embodiments, media replacement is done until the cells are transferred to an alternative growth chamber. In some embodiments, 2/3 of the media is replaced by respiration with fresh media. In some embodiments, alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[00554]** In an embodiment, the second expansion (including expansions referred to as REP) is performed and further comprises a step wherein TILs are selected for superior tumor reactivity. Any selection method known in the art may be used. For example, the methods described in U.S. Patent Application Publication No. 2016/0010058 A1, the disclosures of

which are incorporated herein by reference, may be used for selection of TILs for superior tumor reactivity.

**[00555]** Optionally, a cell viability assay can be performed after the second expansion (including expansions referred to as the REP expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. In some embodiments, TIL samples can be counted and viability determined using a Cellometer K2 automated cell counter (Nexcelom Bioscience, Lawrence, MA). In some embodiments, viability is determined according to the standard Cellometer K2 Image Cytometer Automatic Cell Counter protocol.

**[00556]** In some embodiments, the second expansion (including expansions referred to as REP) of TIL can be performed using T-175 flasks and gas-permeable bags as previously described (Tran KQ, Zhou J, Durflinger KH, et al., **2008**, *J Immunother.*, 31:742–751, and Dudley ME, Wunderlich JR, Shelton TE, et al. 2003, *J Immunother.*, 26:332–342) or gas-permeable G-Rex flasks. In some embodiments, the second expansion is performed using flasks. In some embodiments, the second expansion is performed using gas-permeable G-Rex flasks. In some embodiments, the second expansion is performed in T-175 flasks, and about 1 x $10^6$ TIL are suspended in about 150 mL of media and this is added to each T-175 flask. The TIL are cultured with irradiated (50 Gy) allogeneic PBMC as "feeder" cells at a ratio of 1 to 100 and the cells were cultured in a 1 to 1 mixture of CM and AIM-V medium (50/50 medium), supplemented with 3000 IU/mL of IL-2 and 30 ng/mL of anti-CD3. The T-175 flasks are incubated at 37°C in 5% $CO_2$. In some embodiments, half the media is changed on day 5 using 50/50 medium with 3000 IU/mL of IL-2. In some embodiments, on day 7, cells from 2 T-175 flasks are combined in a 3 L bag and 300 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to the 300 mL of TIL suspension. The number of cells in each bag can be counted every day or two and fresh media can be added to keep the cell count between about 0.5 and about 2.0 x $10^6$ cells/mL.

**[00557]** In some embodiments, the second expansion (including expansions referred to as REP) are performed in 500 mL capacity flasks with 100 $cm^2$ gas-permeable silicon bottoms (G-Rex 100, Wilson Wolf) (Fig. 1), about 5x$10^6$ or 10x$10^6$ TIL are cultured with irradiated allogeneic PBMC at a ratio of 1 to 100 in 400 mL of 50/50 medium, supplemented with 3000 IU/mL of IL-2 and 30 ng/ mL of anti-CD3. The G-Rex 100 flasks are incubated at 37°C in 5% $CO_2$. In some embodiments, on day 5, 250mL of supernatant is removed and placed into

centrifuge bottles and centrifuged at 1500 rpm (491g) for 10 minutes. The TIL pellets can then be resuspended with 150 mL of fresh 50/50 medium with 3000 IU/ mL of IL-2 and added back to the original G-Rex 100 flasks. In embodiments where TILs are expanded serially in G-Rex 100 flasks, on day 7 the TIL in each G-Rex 100 are suspended in the 300 mL of media present in each flask and the cell suspension was divided into three 100 mL aliquots that are used to seed 3 G-Rex 100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to each flask. The G-Rex 100 flasks are incubated at 37°C in 5% $CO_2$ and after 4 days 150 mL of AIM-V with 3000 IU/mL of IL-2 is added to each G-Rex 100 flask. The cells are harvested on day 14 of culture.

**[00558]** The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained in the second expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCRα/β).

**[00559]** In some embodiments, the second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below.

**[00560]** In some embodiments, the second expansion, for example, Step D according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single

bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

1. Feeder Cells and Antigen Presenting Cells

**[00561]** In an embodiment, the second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 1, as well as those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

**[00562]** In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

**[00563]** In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

**[00564]** In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 3000 IU/ml IL-2.

**[00565]** In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/ml OKT3 antibody and 1000-6000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/ml OKT3 antibody and

2000-5000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/ml OKT3 antibody and 2000-4000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/ml OKT3 antibody and 2500-3500 IU/ml IL-2.

**[00566]** In some embodiments, the antigen-presenting feeder cells are PBMCs. In some embodiments, the antigen-presenting feeder cells are artificial antigen-presenting feeder cells. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

**[00567]** In an embodiment, the second expansion procedures described herein require a ratio of about $2.5x10^9$ feeder cells to about $100x10^6$ TILs. In another embodiment, the second expansion procedures described herein require a ratio of about $2.5x10^9$ feeder cells to about $50x10^6$ TILs. In yet another embodiment, the second expansion procedures described herein require about $2.5x10^9$ feeder cells to about $25x10^6$ TILs.

**[00568]** In an embodiment, the second expansion procedures described herein require an excess of feeder cells during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In an embodiment, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

**[00569]** In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

**[00570]** In an embodiment, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

**[00571]** The expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

**[00572]** Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILS is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in International Publication No. WO 2015/189356 and W International Publication No. WO 2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21 and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

### E. STEP E: Harvest TILS

**[00573]** After the second expansion step, cells can be harvested. In some embodiments the TILs are harvested after one, two, three, four or more expansion steps, for example as provided in Figure 1. In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 1.

**[00574]** TILs can be harvested in any appropriate and sterile manner, including for example by centrifugation. Methods for TIL harvesting are well known in the art and any such know methods can be employed with the present process. In some embodiments, TILS are harvest using an automated system.

**[00575]** Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing systems is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term “LOVO cell processing system” also refers to any instrument or device manufactured by any vendor that can pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

**[00576]** In some embodiments, the harvest, for example, Step E according to Figure 1, is performed from a closed system bioreactor. In some embodiments, a closed system is

employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

**[00577]** In some embodiments, Step E according to Figure 1, is performed according to the processes described in Example G. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in Example G is employed.

**[00578]** In some embodiments, TILs are harvested according to the methods described in Example G. In some embodiments, TILs between days 1 and 11 are harvested using the methods as described in Section 8.5 (referred to as the Day 11 TIL harvest in Example G). In some embodiments, TILs between days 12 and 22 are harvested using the methods as described in Section 8.12 (referred to as the Day 22 TIL harvest in Example G).

### F. STEP F: Final Formulation/ Transfer to Infusion Bag

**[00579]** After Steps A through E as provided in an exemplary order in Figure 1 and as outlined in detailed above and herein are complete, cells are transferred to a container for use in administration to a patient. In some embodiments, once a therapeutically sufficient number of TILs are obtained using the expansion methods described above, they are transferred to a container for use in administration to a patient.

**[00580]** In an embodiment, TILs expanded using APCs of the present disclosure are administered to a patient as a pharmaceutical composition. In an embodiment, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded using PBMCs of the present disclosure may be administered by any suitable route as known in the art. In some embodiments, the T-cells are administered as a single intra-arterial or intravenous infusion, which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic.

### G. Optional Cell Medium Components

1. Anti-CD3 Antibodies

**[00581]** In some embodiments, the culture media used in expansion methods described herein (including those referred to as REP, see for example, Figure 1) also includes an anti-CD3 antibody. An anti-CD3 antibody in combination with IL-2 induces T cell activation and

cell division in the TIL population. This effect can be seen with full length antibodies as well as Fab and F(ab')2 fragments, with the former being generally preferred; see, *e.g.,* Tsoukas *et al.*, *J. Immunol.* **1985,** *135,* 1719, hereby incorporated by reference in its entirety.

**[00582]** As will be appreciated by those in the art, there are a number of suitable anti-human CD3 antibodies that find use in the invention, including anti-human CD3 polyclonal and monoclonal antibodies from various mammals, including, but not limited to, murine, human, primate, rat, and canine antibodies. In particular embodiments, the OKT3 anti-CD3 antibody is used (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA).

TABLE 5: Amino acid sequences of muromonab (exemplary OKT-3 antibody)

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:1<br>Muromonab heavy chain | QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY<br>NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA<br>KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL<br>YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN<br>STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE<br>LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:2<br>Muromonab light chain | QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH<br>FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS<br>SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL<br>TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC | 60<br>120<br>180<br>213 |

2. 4-1BB (CD137) AGONISTS

**[00583]** In an embodiment, the TNFRSF agonist is a 4-1BB (CD137) agonist. The 4-1BB agonist may be any 4-1BB binding molecule known in the art. The 4-1BB binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian 4-1BB. The 4-1BB agonists or 4-1BB binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The 4-1BB agonist or 4-1BB binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to 4-1BB. In an embodiment, the 4-1BB agonist is

an antigen binding protein that is a fully human antibody. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a humanized antibody. In some embodiments, 4-1BB agonists for use in the presently disclosed methods and compositions include anti-4-1BB antibodies, human anti-4-1BB antibodies, mouse anti-4-1BB antibodies, mammalian anti-4-1BB antibodies, monoclonal anti-4-1BB antibodies, polyclonal anti-4-1BB antibodies, chimeric anti-4-1BB antibodies, anti-4-1BB adnectins, anti-4-1BB domain antibodies, single chain anti-4-1BB fragments, heavy chain anti-4-1BB fragments, light chain anti-4-1BB fragments, anti-4-1BB fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. Agonistic anti-4-1BB antibodies are known to induce strong immune responses. Lee, *et al.*, *PLOS One* **2013**, *8,* e69677. In a preferred embodiment, the 4-1BB agonist is an agonistic, anti-4-1BB humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line). In an embodiment, the 4-1BB agonist is EU-101 (Eutilex Co. Ltd.), utomilumab, or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof. In a preferred embodiment, the 4-1BB agonist is utomilumab or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof.

**[00584]** In a preferred embodiment, the 4-1BB agonist or 4-1BB binding molecule may also be a fusion protein. In a preferred embodiment, a multimeric 4-1BB agonist, such as a trimeric or hexameric 4-1BB agonist (with three or six ligand binding domains), may induce superior receptor (4-1BBL) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

**[00585]** Agonistic 4-1BB antibodies and fusion proteins are known to induce strong immune responses. In a preferred embodiment, the 4-1BB agonist is a monoclonal antibody or fusion protein that binds specifically to 4-1BB antigen in a manner sufficient to reduce toxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the

4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein which abrogates Fc region functionality.

**[00586]** In some embodiments, the 4-1BB agonists are characterized by binding to human 4-1BB (SEQ ID NO:9) with high affinity and agonistic activity. In an embodiment, the 4-1BB agonist is a binding molecule that binds to human 4-1BB (SEQ ID NO:9). In an embodiment, the 4-1BB agonist is a binding molecule that binds to murine 4-1BB (SEQ ID NO:10). The amino acid sequences of 4-1BB antigen to which a 4-1BB agonist or binding molecule binds are summarized in Table 6.

TABLE 6. Amino acid sequences of 4-1BB antigens.

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:9 human 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Homo sapiens) | MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR 60<br>TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC 120<br>CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE 180<br>PGHSPQIISF FLALTSTALL FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG 240<br>CSCRFPEEEE GGCEL 255 |
| SEQ ID NO:10 murine 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Mus musculus) | MGNNCYNVVV IVLLLVGCEK VGAVQNSCDN CQPGTFCRKY NPVCKSCPPS TFSSIGGQPN 60<br>CNICRVCAGY FRFKKFCSST HNAECECIEG FHCLGPQCTR CEKDCRPGQE LTKQGCKTCS 120<br>LGTFNDQNGT GVCRPWTNCS LDGRSVLKTG TTEKDVVCGP PVVSFSPSTT ISVTPEGGPG 180<br>GHSLQVLTLF LALTSALLLA LIFITLLFSV LKWIRKKFPH IFKQPFKKTT GAAQEEDACS 240<br>CRCPQEEEGG GGGYEL 256 |

**[00587]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds human or murine 4-1BB with a $K_D$ of about 100 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 90 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 80 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 70 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 60 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 50 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 40 pM or lower, or binds human or murine 4-1BB with a $K_D$ of about 30 pM or lower.

**[00588]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $8 \times 10^5$ l/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $8.5 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $9 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $9.5 \times 10^5$ 1/M·s or faster, or binds to human or murine 4-1BB with a $k_{assoc}$ of about $1 \times 10^6$ 1/M·s or faster.

**[00589]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.1 \times 10^{-5}$ 1/s or slower , binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.2 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.3 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.4 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.5 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.6 \times 10^{-5}$ 1/s or slower or binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.7 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.8 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.9 \times 10^{-5}$ 1/s or slower, or binds to human or murine 4-1BB with a $k_{dissoc}$ of about $3 \times 10^{-5}$ 1/s or slower.

**[00590]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with an $IC_{50}$ of about 10 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 9 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 8 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 7 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 6 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 5 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 4 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 3 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 2 nM or lower, or binds to human or murine 4-1BB with an $IC_{50}$ of about 1 nM or lower.

**[00591]** In a preferred embodiment, the 4-1BB agonist is utomilumab, also known as PF-05082566 or MOR-7480, or a fragment, derivative, variant, or biosimilar thereof. Utomilumab is available from Pfizer, Inc. Utomilumab is an immunoglobulin G2-lambda, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor (TNFR) superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of utomilumab are set forth in Table EE. Utomilumab comprises glycosylation sites at Asn59 and Asn292; heavy chain intrachain disulfide bridges at positions 22-96 ($V_H$-$V_L$), 143-199 ($C_H1$-$C_L$), 256-316 ($C_H2$) and 362-420 ($C_H3$); light chain intrachain disulfide bridges at positions 22'-87' ($V_H$-$V_L$) and 136'-195' ($C_H1$-$C_L$); interchain heavy chain-heavy chain disulfide bridges at IgG2A isoform positions 218-218, 219-219, 222-222, and 225-225, at IgG2A/B isoform positions 218-130, 219-219, 222-222, and 225-225, and at IgG2B isoform positions 219-130 (2), 222-222, and 225-225; and interchain heavy chain-light chain disulfide bridges at IgG2A isoform positions 130-213' (2), IgG2A/B

isoform positions 218-213' and 130-213', and at IgG2B isoform positions 218-213' (2). The preparation and properties of utomilumab and its variants and fragments are described in U.S. Patent Nos. 8,821,867; 8,337,850; and 9,468,678, and International Patent Application Publication No. WO 2012/032433 A1, the disclosures of each of which are incorporated by reference herein. Preclinical characteristics of utomilumab are described in Fisher, *et al.*, *Cancer Immunolog. & Immunother.* **2012,** *61,* 1721-33. Current clinical trials of utomilumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02444793, NCT01307267, NCT02315066, and NCT02554812.

**[00592]** In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:11 and a light chain given by SEQ ID NO:12. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively.

**[00593]** In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of utomilumab. In an embodiment, the 4-1BB agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:13, and the 4-1BB agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:14, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$

regions that are each at least 97% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14.

**[00594]** In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:18, SEQ ID NO:19, and SEQ ID NO:20, respectively, and conservative amino acid substitutions thereof.

**[00595]** In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to utomilumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the biosimilar is provided as a composition which further

comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab.

TABLE 7. Amino acid sequences for 4-1BB agonist antibodies related to utomilumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:11 heavy chain for utomilumab | EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMGK IYPGDSYTNY<br>SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCARGY GIFDYWGQGT LVTVSSASTK<br>GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVHTFP AVLQSSGLYS<br>LSSVVTVPSS NFGTQTYTCN VDHKPSNTKV DKTVERKCCV ECPPCPAPPV AGPSVFLFPP<br>KPKDTLMISR TPEVTCVVVD VSHEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTFRVVSV<br>LTVVHQDWLN GKEYKCKVSN KGLPAPIEKT ISKTKGQPRE PQVYTLPPSR EEMTKNQVSL<br>TCLVKGFYPS DIAVEWESNG QPENNYKTTP PMLDSDGSFF LYSKLTVDKS RWQQGNVFSC<br>SVMHEALHNH YTQKSLSLSP G | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>441 |
| SEQ ID NO:12 light chain for utomilumab | SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER<br>FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVLGQ PKAAPSVTLF<br>PPSSEELQAN KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTPSKQS NNKYAASSYL<br>SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS | 60<br>120<br>180<br>214 |
| SEQ ID NO:13 heavy chain variable region for utomilumab | EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMG KIYPGDSYTN<br>YSPSFQGQVT ISADKSISTA YLQWSSLKAS DTAMYYCARG YGIFDYWGQ GTLVTVSS | 60<br>118 |
| SEQ ID NO:14 light chain variable region for utomilumab | SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER<br>FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVL | 60<br>108 |
| SEQ ID NO:15 heavy chain CDR1 for utomilumab | STYWIS | 6 |
| SEQ ID NO:16 heavy chain CDR2 for utomilumab | KIYPGDSYTN YSPSFQG | 17 |
| SEQ ID NO:17 heavy chain CDR3 for utomilumab | RGYGIFDY | 8 |
| SEQ ID NO:18 light chain CDR1 for utomilumab | SGDNIGDQYA H | 11 |
| SEQ ID NO:19 light chain CDR2 for utomilumab | QDKNRPS | 7 |
| SEQ ID NO:20 light chain CDR3 for utomilumab | ATYTGFGSLA V | 11 |

**[00596]** In a preferred embodiment, the 4-1BB agonist is the monoclonal antibody urelumab, also known as BMS-663513 and 20H4.9.h4a, or a fragment, derivative, variant, or biosimilar thereof. Urelumab is available from Bristol-Myers Squibb, Inc., and Creative Biolabs, Inc. Urelumab is an immunoglobulin G4-kappa, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of urelumab are set forth in Table EE. Urelumab comprises N-glycosylation sites at positions 298 (and 298'); heavy chain intrachain disulfide bridges at positions 22-95 ($V_H$-$V_L$), 148-204 ($C_H1$-$C_L$), 262-322 ($C_H2$) and 368-426 ($C_H3$) (and at positions 22''-95'', 148''-204'', 262''-322'', and 368''-426''); light chain intrachain disulfide bridges at positions 23'-88' ($V_H$-$V_L$) and 136'-196'

($C_H1$-$C_L$) (and at positions 23'''-88''' and 136'''-196'''); interchain heavy chain-heavy chain disulfide bridges at positions 227-227'' and 230-230''; and interchain heavy chain-light chain disulfide bridges at 135-216' and 135''-216'''. The preparation and properties of urelumab and its variants and fragments are described in U.S. Patent Nos. 7,288,638 and 8,962,804, the disclosures of which are incorporated by reference herein. The preclinical and clinical characteristics of urelumab are described in Segal, *et al.*, *Clin. Cancer Res.* **2016,** *available at* http:/dx.doi.org/ 10.1158/1078-0432.CCR-16-1272. Current clinical trials of urelumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT01775631, NCT02110082, NCT02253992, and NCT01471210.

**[00597]** In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:21 and a light chain given by SEQ ID NO:22. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively.

**[00598]** In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of urelumab. In an embodiment, the 4-1BB agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:23, and the 4-1BB agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:24, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:23

and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24.

**[00599]** In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:25, SEQ ID NO:26, and SEQ ID NO:27, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30, respectively, and conservative amino acid substitutions thereof.

**[00600]** In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to urelumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab.

In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab.

TABLE 8: Amino acid sequences for 4-1BB agonist antibodies related to urelumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:21 heavy chain for urelumab | QVQLQQWGAG LLKPSETLSL TCAVYGGSFS GYYWSWIRQS PEKGLEWIGE INHGGYVTYN<br>PSLESRVTIS VDTSKNQFSL KLSSVTAADT AVYYCARDYG PGNYDWYFDL WGRGTLVTVS<br>SASTKGPSVF PLAPCSRSTS ESTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS<br>SGLYSLSSVV TVPSSSLGTK TYTCNVDHKP SNTKVDKRVE SKYGPPCPPC PAPEFLGGPS<br>VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST<br>YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT<br>KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE<br>GNVFSCSVMH EALHNHYTQK SLSLSLGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>448 |
| SEQ ID NO:22 light chain for urelumab | EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPALTF CGGTKVEIKR TVAAPSVFIF<br>PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYSLSST<br>LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC | 60<br>120<br>180<br>216 |
| SEQ ID NO:23 variable heavy chain for urelumab | MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YYWSWIRQSP<br>EKGLEWIGEI NHGGYVTYNP SLESRVTISV DTSKNQFSLK LSSVTAADTA VYYCARDYGP | 60<br>120 |
| SEQ ID NO:24 variable light chain for urelumab | MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP<br>GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ | 60<br>110 |
| SEQ ID NO:25 heavy chain CDR1 for urelumab | GYYWS | 5 |
| SEQ ID NO:26 heavy chain CDR2 for urelumab | EINHGGYVTY NPSLES | 16 |
| SEQ ID NO:27 heavy chain CDR3 for urelumab | DYGPGNYDWY FDL | 13 |
| SEQ ID NO:28 light chain CDR1 for urelumab | RASQSVSSYL A | 11 |
| SEQ ID NO:29 light chain CDR2 for urelumab | DASNRAT | 7 |
| SEQ ID NO:30 light chain CDR3 for urelumab | QQRSDWPPAL T | 11 |

**[00601]** In an embodiment, the 4-1BB agonist is selected from the group consisting of 1D8, 3Elor, 4B4 (BioLegend 309809), H4-1BB-M127 (BD Pharmingen 552532), BBK2 (Thermo Fisher MS621PABX), 145501 (Leinco Technologies B591), the antibody produced by cell line deposited as ATCC No. HB-11248 and disclosed in U.S. Patent No. 6,974,863, 5F4 (BioLegend 31 1503), C65-485 (BD Pharmingen 559446), antibodies disclosed in U.S. Patent Application Publication No. US 2005/0095244, antibodies disclosed in U.S. Patent No. 7,288,638 (such as 20H4.9-IgGl (BMS-663031)), antibodies disclosed in U.S. Patent No. 6,887,673 (such as 4E9 or BMS-554271), antibodies disclosed in U.S. Patent No. 7,214,493, antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in U.S. Patent No. 6,905,685 (such as 4E9 or BMS-554271),

antibodies disclosed in U.S. Patent No. 6,362,325 (such as 1D8 or BMS-469492; 3H3 or BMS-469497; or 3El), antibodies disclosed in U.S. Patent No. 6,974,863 (such as 53A2); antibodies disclosed in U.S. Patent No. 6,210,669 (such as 1D8, 3B8, or 3El), antibodies described in U.S. Patent No. 5,928,893, antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in International Patent Application Publication Nos. WO 2012/177788, WO 2015/119923, and WO 2010/042433, and fragments, derivatives, conjugates, variants, or biosimilars thereof, wherein the disclosure of each of the foregoing patents or patent application publications is incorporated by reference here.

**[00602]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein described in International Patent Application Publication Nos. WO 2008/025516 A1, WO 2009/007120 A1, WO 2010/003766 A1, WO 2010/010051 A1, and WO 2010/078966 A1; U.S. Patent Application Publication Nos. US 2011/0027218 A1, US 2015/0126709 A1, US 2011/0111494 A1, US 2015/0110734 A1, and US 2015/0126710 A1; and U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

**[00603]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof (see, Figure 50). In structures I-A and I-B, the cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from *e.g.*, 4-1BBL or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second triavelent protein through IgG1-Fc (including $C_H3$ and $C_H2$ domains) is then used to link two of the trivalent proteins together through disulfide bonds (small elongated ovals), stabilizing the structure and providing an agonists capable of bringing together the intracellular signaling domains of the six receptors and signaling proteins to form a signaling complex. The TNFRSF binding domains denoted as cylinders may be scFv domains comprising, *e.g.*, a $V_H$ and a $V_L$ chain connected by a linker that may comprise hydrophilic residues and Gly and Ser sequences for flexibility, as well as Glu and Lys for solubility. Any scFv domain design may be used, such as those described in de Marco, *Microbial Cell Factories*, **2011,** *10*, 44; Ahmad, *et al.*, *Clin. & Dev. Immunol.* **2012,** 980250; Monnier, *et al.*, *Antibodies,* **2013,** *2,* 193-208; or in references incorporated elsewhere herein. Fusion protein structures of this

form are described in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

**[00604]** Amino acid sequences for the other polypeptide domains of structure I-A are given in Table GG. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides.

TABLE 9: Amino acid sequences for TNFRSF fusion proteins, including 4-1BB fusion proteins, with C-terminal Fc-antibody fragment fusion protein design (structure I-A).

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:31<br>Fc domain | KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW<br>YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS<br>KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV<br>LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>230 |
| SEQ ID NO:32<br>linker | GGPGSSKSCD KTHTCPPCPA PE | 22 |
| SEQ ID NO:33<br>linker | GGSGSSKSCD KTHTCPPCPA PE | 22 |
| SEQ ID NO:34<br>linker | GGPGSSSSSS SKSCDKTHTC PPCPAPE | 27 |
| SEQ ID NO:35<br>linker | GGSGSSSSSS SKSCDKTHTC PPCPAPE | 27 |
| SEQ ID NO:36<br>linker | GGPGSSSSSS SSSKSCDKTH TCPPCPAPE | 29 |
| SEQ ID NO:37<br>linker | GGSGSSSSSS SSSKSCDKTH TCPPCPAPE | 29 |
| SEQ ID NO:38<br>linker | GGPGSSGSGS SDKTHTCPPC PAPE | 24 |
| SEQ ID NO:39<br>linker | GGPGSSGSGS DKTHTCPPCP APE | 23 |
| SEQ ID NO:40<br>linker | GGPSSSGSDK THTCPPCPAP E | 21 |
| SEQ ID NO:41<br>linker | GGSSSSSSSS GSDKTHTCPP CPAPE | 25 |

**[00605]** Amino acid sequences for the other polypeptide domains of structure I-B are given in Table HH. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

TABLE 10: Amino acid sequences for TNFRSF fusion proteins, including 4-1BB fusion proteins, with N-terminal Fc-antibody fragment fusion protein design (structure I-B).

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:42<br>Fc domain | METDTLLLWV LLLWVPAGNG DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT<br>CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK<br>CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE | 60<br>120<br>180 |

| | | |
|---|---|---|
| | WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG | 240<br>246 |
| SEQ ID NO:43 linker | SGSGSGSGSG S | 11 |
| SEQ ID NO:44 linker | SSSSSSGSGS GS | 12 |
| SEQ ID NO:45 linker | SSSSSSGSGS GSGSGS | 16 |

**[00606]** In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains selected from the group consisting of a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain of urelumab, a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table GG, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

**[00607]** In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:46. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a soluble 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:47.

**[00608]** In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the $V_H$ and $V_L$ sequences given in Table 11, wherein the $V_H$ and $V_L$ domains are connected by a linker.

TABLE 11: Additional polypeptide domains useful as 4-1BB binding domains in fusion proteins or as scFv 4-1BB agonist antibodies.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:46 4-1BBL | MEYASDASLD PEAPWPPAPR ARACRVLPWA LVAGLLLLLL LAAACAVFLA CPWAVSGARA<br>SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL<br>TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAALA<br>LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ GATVLGLFRV<br>TPEIPAGLPS PRSE | 60<br>120<br>180<br>240<br>254 |
| SEQ ID NO:47 4-1BBL soluble domain | LRQGMFAQLV AQNVLLIDGP LSWYSDPGLA GVSLTGGLSY KEDTKELVVA KAGVYYVFFQ<br>LELRRVVAGE GSGSVSLALH LQPLRSAAGA AALALTVDLP PASSEARNSA FGFQGRLLHL<br>SAGQRLGVHL HTEARARHAW QLTQGATVLG LFRVTPEIPA GLPSPRSE | 60<br>120<br>168 |
| SEQ ID NO:48 variable heavy chain for 4B4-1-1 version 1 | QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY<br>NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVS | 60<br>118 |
| SEQ ID NO:49 variable light chain for 4B4-1-1 version 1 | DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS<br>RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIK | 60<br>107 |
| SEQ ID NO:50 variable heavy chain for 4B4-1-1 version 2 | QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY<br>NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVSA | 60<br>119 |
| SEQ ID NO:51 variable light chain for 4B4-1-1 version 2 | DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS<br>RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIKR | 60<br>108 |
| SEQ ID NO:52 variable heavy chain for H39E3-2 | MDWTWRILFL VAAATGAHSE VQLVESGGGL VQPGGSLRLS CAASGFTFSD YWMSWVRQAP<br>GKGLEWVADI KNDGSYTNYA PSLTNRFTIS RDNAKNSLYL QMNSLRAEDT AVYYCARELT | 60<br>120 |
| SEQ ID NO:53 variable light chain for H39E3-2 | MEAPAQLLFL LLLWLPDTTG DIVMTQSPDS LAVSLGERAT INCKSSQSLL SSGNQKNYL<br>WYQQKPGQPP KLLIYYASTR QSGVPDRFSG SGSGTDFTLT ISSLQAEDVA | 60<br>110 |

**[00609]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain, wherein each of the soluble 4-1BB domains lacks a stalk region (which contributes to trimerisation and provides a certain distance to the cell membrane, but is not part of the 4-1BB binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

**[00610]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain,

(iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein each TNF superfamily cytokine domain is a 4-1BB binding domain.

**[00611]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic scFv antibody comprising any of the foregoing $V_H$ domains linked to any of the foregoing $V_L$ domains.

**[00612]** In an embodiment, the 4-1BB agonist is BPS Bioscience 4-1BB agonist antibody catalog no. 79097-2, commercially available from BPS Bioscience, San Diego, CA, USA. In an embodiment, the 4-1BB agonist is Creative Biolabs 4-1BB agonist antibody catalog no. MOM-18179, commercially available from Creative Biolabs, Shirley, NY, USA.

3. OX40 (CD134) AGONISTS

**[00613]** In an embodiment, the TNFRSF agonist is an OX40 (CD134) agonist. The OX40 agonist may be any OX40 binding molecule known in the art. The OX40 binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian OX40. The OX40 agonists or OX40 binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The OX40 agonist or OX40 binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to OX40. In an embodiment, the OX40 agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the OX40 agonist is an antigen binding protein that is a humanized antibody. In some embodiments, OX40 agonists for use in the presently disclosed methods and compositions include anti-OX40 antibodies, human anti-OX40 antibodies, mouse anti-OX40 antibodies, mammalian anti-OX40 antibodies, monoclonal anti-OX40 antibodies, polyclonal anti-OX40 antibodies, chimeric anti-OX40 antibodies, anti-OX40 adnectins, anti-OX40 domain antibodies, single chain anti-OX40 fragments, heavy chain anti-OX40 fragments, light chain anti-OX40 fragments, anti-OX40 fusion proteins, and fragments, derivatives, conjugates, variants, or

biosimilars thereof. In a preferred embodiment, the OX40 agonist is an agonistic, anti-OX40 humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line).

**[00614]** In a preferred embodiment, the OX40 agonist or OX40 binding molecule may also be a fusion protein. OX40 fusion proteins comprising an Fc domain fused to OX40L are described, for example, in Sadun, *et al.*, *J. Immunother.* **2009,** *182,* 1481-89. In a preferred embodiment, a multimeric OX40 agonist, such as a trimeric or hexameric OX40 agonist (with three or six ligand binding domains), may induce superior receptor (OX40L) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

**[00615]** Agonistic OX40 antibodies and fusion proteins are known to induce strong immune responses. Curti, *et al.*, *Cancer Res.* **2013**, *73,* 7189-98. In a preferred embodiment, the OX40 agonist is a monoclonal antibody or fusion protein that binds specifically to OX40 antigen in a manner sufficient to reduce toxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein which abrogates Fc region functionality.

**[00616]** In some embodiments, the OX40 agonists are characterized by binding to human OX40 (SEQ ID NO:54) with high affinity and agonistic activity. In an embodiment, the OX40 agonist is a binding molecule that binds to human OX40 (SEQ ID NO:54). In an embodiment, the OX40 agonist is a binding molecule that binds to murine OX40 (SEQ ID NO:55). The amino acid sequences of OX40 antigen to which an OX40 agonist or binding molecule binds are summarized in Table 12.

TABLE 12: Amino acid sequences of OX40 antigens.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:54<br>human OX40<br>(Homo sapiens) | MCVGARRLGR GPCAALLLLG LGLSTVTGLH CVGDTYPSND RCCHECRPGN GMVSRCSRSQ<br>NTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSERKQLCT ATQDTVCRCR AGTQPLDSYK<br>PGVDCAPCPP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQ<br>GPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL<br>RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI | 60<br>120<br>180<br>240<br>277 |
| SEQ ID NO:55<br>murine OX40<br>(Mus musculus) | MYVWVQQPTA LLLLGLTLGV TARRLNCVKH TYPSGHKCCR ECQPGHGMVS RCDHTRDTLC<br>HPCETGFYNE AVNYDTCKQC TQCNHRSGSE LKQNCTPTQD TVCRCRPGTQ PRQDSGYKLG<br>VDCVPCPPGH FSPGNNQACK PWTNCTLSGK QTRHPASDSL DAVCEDRSLL ATLLWETQRP<br>TFRPTTVQST TVWPRTSELP SPPTLVTPEG PAFAVLLGLG LGLLAPLTVL LALYLLRKAW<br>RLPNTPKPCW GNSFRTPIQE EHTDAHFTLA KI | 60<br>120<br>180<br>240<br>272 |

**[00617]** In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds human or murine OX40 with a $K_D$ of about 100 pM or lower, binds human or murine OX40 with a $K_D$ of about 90 pM or lower, binds human or murine OX40 with a $K_D$ of about 80 pM or lower, binds human or murine OX40 with a $K_D$ of about 70 pM or lower, binds human or murine OX40 with a $K_D$ of about 60 pM or lower, binds human or murine OX40 with a $K_D$ of about 50 pM or lower, binds human or murine OX40 with a $K_D$ of about 40 pM or lower, or binds human or murine OX40 with a $K_D$ of about 30 pM or lower.

**[00618]** In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $8 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $8.5 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $9 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $9.5 \times 10^5$ 1/M·s or faster, or binds to human or murine OX40 with a $k_{assoc}$ of about $1 \times 10^6$ 1/M·s or faster.

**[00619]** In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a $k_{dissoc}$ of about $2 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.1 \times 10^{-5}$ 1/s or slower , binds to human or murine OX40 with a $k_{dissoc}$ of about $2.2 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.3 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.4 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.5 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.6 \times 10^{-5}$ 1/s or slower or binds to human or murine OX40 with a $k_{dissoc}$ of about $2.7 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.8 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.9 \times 10^{-5}$ 1/s or slower, or binds to human or murine OX40 with a $k_{dissoc}$ of about $3 \times 10^{-5}$ 1/s or slower.

**[00620]** In some embodiments, the compositions, processes and methods described include OX40 agonist that binds to human or murine OX40 with an $IC_{50}$ of about 10 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 9 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 8 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 7 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 6 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 5 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 4 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 3 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 2 nM or lower, or binds to human or murine OX40 with an $IC_{50}$ of about 1 nM or lower.

**[00621]** In some embodiments, the OX40 agonist is tavolixizumab, also known as MEDI0562 or MEDI-0562. Tavolixizumab is available from the MedImmune subsidiary of AstraZeneca, Inc. Tavolixizumab is immunoglobulin G1-kappa, anti-[*Homo sapiens* TNFRSF4 (tumor necrosis factor receptor (TNFR) superfamily member 4, OX40, CD134)], humanized and chimeric monoclonal antibody. The amino acid sequences of tavolixizumab are set forth in Table KK. Tavolixizumab comprises N-glycosylation sites at positions 301 and 301'', with fucosylated complex bi-antennary CHO-type glycans; heavy chain intrachain disulfide bridges at positions 22-95 ($V_H$-$V_L$), 148-204 ($C_H1$-$C_L$), 265-325 ($C_H2$) and 371-429 ($C_H3$) (and at positions 22''-95'', 148''-204'', 265''-325'', and 371''-429''); light chain intrachain disulfide bridges at positions 23'-88' ($V_H$-$V_L$) and 134'-194' ($C_H1$-$C_L$) (and at positions 23'''-88''' and 134'''-194'''); interchain heavy chain-heavy chain disulfide bridges at positions 230-230'' and 233-233''; and interchain heavy chain-light chain disulfide bridges at 224-214' and 224''-214'''. Current clinical trials of tavolixizumab in a variety of solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02318394 and NCT02705482.

**[00622]** In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:56 and a light chain given by SEQ ID NO:57. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist

comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively.

**[00623]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of tavolixizumab. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:58, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:59, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, an OX40 agonist comprises an scFv antibody comprising $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59.

**[00624]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:63, SEQ ID NO:64, and SEQ ID NO:65, respectively, and conservative amino acid substitutions thereof.

**[00625]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to tavolixizumab. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or

reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab.

TABLE 13: Amino acid sequences for OX40 agonist antibodies related to tavolixizumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:56 heavy chain for tavolixizumab | QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN<br>PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVTVS<br>SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS<br>SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPELLG<br>GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY<br>NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE<br>EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR<br>WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>451 |
| SEQ ID NO:57 light chain for tavolixizumab | DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS<br>RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKRTV AAPSVFIFPP<br>SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT<br>LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC | 60<br>120<br>180<br>214 |
| SEQ ID NO:58 heavy chain variable region for tavolixizumab | QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN<br>PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVT | 60<br>118 |
| SEQ ID NO:59 light chain variable region for tavolixizumab | DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS<br>RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKR | 60<br>108 |
| SEQ ID NO:60 heavy chain CDR1 for tavolixizumab | GSFSSGYWN | 9 |

| | | |
|---|---|---|
| SEQ ID NO:61 heavy chain CDR2 for tavolixizumab | YIGYISYNGI TYH | 13 |
| SEQ ID NO:62 heavy chain CDR3 for tavolixizumab | RYKYDYDGGH AMDY | 14 |
| SEQ ID NO:63 light chain CDR1 for tavolixizumab | QDISNYLN | 8 |
| SEQ ID NO:64 light chain CDR2 for tavolixizumab | LLIYYTSKLH S | 11 |
| SEQ ID NO:65 light chain CDR3 for tavolixizumab | QQGSALPW | 8 |

**[00626]** In some embodiments, the OX40 agonist is 11D4, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 11D4 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 11D4 are set forth in Table LL.

**[00627]** In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:66 and a light chain given by SEQ ID NO:67. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively.

**[00628]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 11D4. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:68, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:69, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises

$V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively.

**[00629]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:72, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:73, SEQ ID NO:74, and SEQ ID NO:75, respectively, and conservative amino acid substitutions thereof.

**[00630]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 11D4. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product,

wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4.

TABLE 14: Amino acid sequences for OX40 agonist antibodies related to 11D4.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:66 heavy chain for 11D4 | EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSSAS<br>TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL<br>YSLSSVVTVP SSNFGTQTYT CNVDHKPSNT KVDKTVERKC CVECPPCPAP PVAGPSVFLF<br>PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTFRVV<br>SVLTVVHQDW LNGKEYKCKV SNKGLPAPIE KTISKTKGQP REPQVYTLPP SREEMTKNQV<br>SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPMLDSDGS FFLYSKLTVD KSRWQQGNVF<br>SCSVMHEALH NHYTQKSLSL SPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>444 |
| SEQ ID NO:67 light chain for 11D4 | DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS<br>RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIKRTV AAPSVFIFPP<br>SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT<br><br>LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC | 60<br>120<br>180<br><br>214 |
| SEQ ID NO:68 heavy chain variable region for 11D4 | EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSS | 60<br>118 |
| SEQ ID NO:69 light chain variable region for 11D4 | DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS<br>RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIK | 60<br>107 |
| SEQ ID NO:70 heavy chain CDR1 for 11D4 | SYSMN | 5 |
| SEQ ID NO:71 heavy chain CDR2 for 11D4 | YISSSSSTID YADSVKG | 17 |
| SEQ ID NO:72 heavy chain CDR3 for 11D4 | ESGWYLFDY | 9 |
| SEQ ID NO:73 light chain CDR1 for 11D4 | RASQGISSWL A | 11 |
| SEQ ID NO:74 light chain CDR2 for 11D4 | AASSLQS | 7 |
| SEQ ID NO:75 light chain CDR3 for 11D4 | QQYNSYPPT | 9 |

**[00631]** In some embodiments, the OX40 agonist is 18D8, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 18D8 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 18D8 are set forth in Table MM.

**[00632]** In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:76 and a light chain given by SEQ ID NO:77. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively, or antigen binding fragments, Fab fragments, single-chain variable

fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively.

**[00633]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 18D8. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:78, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:79, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively.

**[00634]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:80, SEQ ID NO:81, and SEQ ID NO:82, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:83, SEQ ID NO:84, and SEQ ID NO:85, respectively, and conservative amino acid substitutions thereof.

**[00635]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 18D8. In an embodiment,

the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8.

TABLE 15: Amino acid sequences for OX40 agonist antibodies related to 18D8.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:76 heavy chain for 18D8 | EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV<br>TVSSASTKGP SVFPLAPCSR STSESTAALG CLVKDYFPEP VTVSWNSGAL TSGVHTFPAV<br>LQSSGLYSLS SVVTVPSSNF GTQTYTCNVD HKPSNTKVDK TVERKCCVEC PPCPAPPVAG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVQFNW YVDGVEVHNA KTKPREEQFN<br>STFRVVSVLT VVHQDWLNGK EYKCKVSNKG LPAPIEKTIS KTKGQPREPQ VYTLPPSREE<br>MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPM LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:77 light chain for 18D8 | EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIKRTVA APSVFIFPPS<br>DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL<br>SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC | 60<br>120<br>180<br>213 |
| SEQ ID NO:78 heavy chain variable region for 18D8 | EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV<br>TVSS | 60<br>120<br>124 |
| SEQ ID NO:79 light chain variable region for 18D8 | EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIK | 60<br>106 |

| | | |
|---|---|---|
| SEQ ID NO:80 heavy chain CDR1 for 18D8 | DYAMH | 5 |
| SEQ ID NO:81 heavy chain CDR2 for 18D8 | GISWNSGSIG YADSVKG | 17 |
| SEQ ID NO:82 heavy chain CDR3 for 18D8 | DQSTADYYFY YGMDV | 15 |
| SEQ ID NO:83 light chain CDR1 for 18D8 | RASQSVSSYL A | 11 |
| SEQ ID NO:84 light chain CDR2 for 18D8 | DASNRAT | 7 |
| SEQ ID NO:85 light chain CDR3 for 18D8 | QQRSNWPT | 8 |

**[00636]** In some embodiments, the OX40 agonist is Hu119-122, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu119-122 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu119-122 are set forth in Table NN.

**[00637]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu119-122. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:86, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:87, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively.

**[00638]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:90, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:91, SEQ ID NO:92, and SEQ ID NO:93, respectively, and conservative amino acid substitutions thereof.

**[00639]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu119-122. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122.

TABLE 16: Amino acid sequences for OX40 agonist antibodies related to Hu119-122.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:86 heavy chain variable region for Hu119-122 | EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY<br>PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS | 60<br>120 |
| SEQ ID NO:87 light chain variable region for Hu119-122 | EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES<br>GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K | 60<br>111 |
| SEQ ID NO:88 heavy chain CDRl for Hu119-122 | SHDMS | 5 |
| SEQ ID NO:89 heavy chain CDR2 for Hu119-122 | AINSDGGSTY YPDTMER | 17 |

| | | |
|---|---|---|
| SEQ ID NO:90 heavy chain CDR3 for Hu119-122 | HYDDYYAWFA Y | 11 |
| SEQ ID NO:91 light chain CDR1 for Hu119-122 | RASKSVSTSG YSYMH | 15 |
| SEQ ID NO:92 light chain CDR2 for Hu119-122 | LASNLES | 7 |
| SEQ ID NO:93 light chain CDR3 for Hu119-122 | QHSRELPLT | 9 |

**[00640]** In some embodiments, the OX40 agonist is Hu106-222, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu106-222 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu106-222 are set forth in Table OO.

**[00641]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu106-222. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:94, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:95, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively.

**[00642]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:99, SEQ ID NO:100, and SEQ ID NO:101, respectively, and conservative amino acid substitutions thereof.

**[00643]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu106-222. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222.

TABLE 17: Amino acid sequences for OX40 agonist antibodies related to Hu106-222.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:94 heavy chain variable region for Hu106-222 | QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY<br>ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV<br>SS | 60<br>120<br>122 |
| SEQ ID NO:95 light chain variable region for Hu106-222 | DIQMTQSPSS LSASVGDRVT ITCKASQDVS TAVAWYQQKP GKAPKLLIYS ASYLYTGVPS<br>RFSGSGSGTD FTFTISSLQP EDIATYYCQQ HYSTPRTFGQ GTKLEIK | 60<br>107 |
| SEQ ID NO:96 heavy chain CDR1 for Hu106-222 | DYSMH | 5 |
| SEQ ID NO:97 heavy chain CDR2 for Hu106-222 | WINTETGEPT YADDFKG | 17 |

| | | |
|---|---|---|
| SEQ ID NO:98 heavy chain CDR3 for Hu106-222 | PYYDYVSYYA MDY | 13 |
| SEQ ID NO:99 light chain CDR1 for Hu106-222 | KASQDVSTAV A | 11 |
| SEQ ID NO:100 light chain CDR2 for Hu106-222 | SASYLYT | 7 |
| SEQ ID NO:101 light chain CDR3 for Hu106-222 | QQHYSTPRT | 9 |

**[00644]** In some embodiments, the OX40 agonist antibody is MEDI6469 (also referred to as 9B12). MEDI6469 is a murine monoclonal antibody. Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585. In some embodiments the OX40 agonist is an antibody produced by the 9B12 hybridoma, deposited with Biovest Inc. (Malvern, MA, USA), as described in Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585, the disclosure of which is hereby incorporated by reference in its entirety. In some embodiments, the antibody comprises the CDR sequences of MEDI6469. In some embodiments, the antibody comprises a heavy chain variable region sequence and/or a light chain variable region sequence of MEDI6469.

**[00645]** In an embodiment, the OX40 agonist is L106 BD (Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises the CDRs of antibody L106 (BD Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody L106 (BD Pharmingen Product #340420). In an embodiment, the OX40 agonist is ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises the CDRs of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In an embodiment, the OX40 agonist is the murine monoclonal antibody anti-mCD134/mOX40 (clone OX86), commercially available from InVivoMAb, BioXcell Inc, West Lebanon, NH.

**[00646]** In an embodiment, the OX40 agonist is selected from the OX40 agonists described in International Patent Application Publication Nos. WO 95/12673, WO 95/21925, WO 2006/121810, WO 2012/027328, WO 2013/028231, WO 2013/038191, and WO 2014/148895; European Patent Application EP 0672141; U.S. Patent Application Publication Nos. US 2010/136030, US 2014/377284, US 2015/190506, and US 2015/132288 (including clones 20E5 and 12H3); and U.S. Patent Nos. 7,504,101, 7,550,140, 7,622,444, 7,696,175,

7,960,515, 7,961,515, 8,133,983, 9,006,399, and 9,163,085, the disclosure of each of which is incorporated herein by reference in its entirety.

**[00647]** In an embodiment, the OX40 agonist is an OX40 agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof. The properties of structures I-A and I-B are described above and in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein. Amino acid sequences for the polypeptide domains of structure I-A are given in Table GG. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides. Likewise, amino acid sequences for the polypeptide domains of structure I-B are given in Table HH. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

**[00648]** In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains selected from the group consisting of a variable heavy chain and variable light chain of tavolixizumab, a variable heavy chain and variable light chain of 11D4, a variable heavy chain and variable light chain of 18D8, a variable heavy chain and variable light chain of Hu119-122, a variable heavy chain and variable light chain of Hu106-222, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table OO, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

**[00649]** In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising an OX40L sequence. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:102. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a soluble OX40L sequence. In an

embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:103. In an embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:104.

**[00650]** In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the $V_H$ and $V_L$ sequences given in Table 18, wherein the $V_H$ and $V_L$ domains are connected by a linker.

TABLE 18: Additional polypeptide domains useful as OX40 binding domains in fusion proteins (*e.g.*, structures I-A and I-B) or as scFv OX40 agonist antibodies.

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:102<br>OX40L | MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL QVSHRYPRIQ 60<br>SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYFS QEVNISLHYQ 120<br>KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF 180<br>CVL 183 |

| | | |
|---|---|---|
| SEQ ID NO:103 OX40L soluble domain | SHRYPRIQSI KVQFTEYKKE KGFILTSQKE DEIMKVQNNS VIINCDGFYL ISLKGYFSQE<br>VNISLHYQKD EEPLFQLKKV RSVNSLMVAS LTYKDKVYLN VTTDNTSLDD FHVNGGELIL<br>IHQNPGEFCV L | 60<br>120<br>131 |
| SEQ ID NO:104 OX40L soluble domain (alternative) | YPRIQSIKVQ FTEYKKEKGF ILTSQKEDEI MKVQNNSVII NCDGFYLISL KGYFSQEVNI<br>SLHYQKDEEP LFQLKKVRSV NSLMVASLTY KDKVYLNVTT DNTSLDDFHV NGGELILIHQ<br>NPGEFCVL | 60<br>120<br>128 |
| SEQ ID NO:105 variable heavy chain for 008 | EVQLVESGGG LVQPGGSLRL SCAASGFTFS NYTMNWVRQA PGKGLEWVSA ISGSGGSTYY<br>ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YSQVHYALDY WGQGTLVTVS | 60<br>120 |
| SEQ ID NO:106 variable light chain for 008 | DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA<br>SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK | 60<br>108 |
| SEQ ID NO:107 variable heavy chain for 011 | EVQLVESGGG VVQPGRSLRL SCAASGFTFS DYTMNWVRQA PGKGLEWVSS ISGGSTYYAD<br>SRKGRFTISR DNSKNTLYLQ MNNLRAEDTA VYYCARDRYF RQQNAFDYWG QGTLVTVSSA | 60<br>120 |
| SEQ ID NO:108 variable light chain for 011 | DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA<br>SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK | 60<br>108 |
| SEQ ID NO:109 variable heavy chain for 021 | EVQLVESGGG LVQPRGSLRL SCAASGFTFS SYAMNWVRQA PGKGLEWVAV ISYDGSNKYY<br>ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YITLPNALDY WGQGTLVTVS | 60<br>120 |
| SEQ ID NO:110 variable light chain for 021 | DIQMTQSPVS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKPGQSPQ LLIYLGSNRA<br>SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYKSNP PTFGQGTK | 60<br>108 |
| SEQ ID NO:111 variable heavy chain for 023 | EVQLVESGGG LVHPGGSLRL SCAGSGFTFS SYAMHWVRQA PGKGLEWVSA IGTGGGTYYA<br>DSVMGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCARYDN VMGLYWFDYW GQGTLVTVSS | 60<br>120 |
| SEQ ID NO:112 variable light chain for 023 | EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPAFGG GTKVEIKR | 60<br>108 |
| SEQ ID NO:113 heavy chain variable region | EVQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY INPYNDGTKY<br>NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCANYY GSSLSMDYWG QGTSVTVSS | 60<br>119 |
| SEQ ID NO:114 light chain variable region | DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS<br>RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTLPWTFGG GTKLEIKR | 60<br>108 |
| SEQ ID NO:115 heavy chain variable region | EVQLQQSGPE LVKPGASVKI SCKTSGYTFK DYTMHWVKQS HGKSLEWIGG IYPNNGGSTY<br>NQNFKDKATL TVDKSSSTAY MEFRSLTSED SAVYYCARMG YHGPHLDFDV WGAGTTVTVS<br>P | 60<br>120<br>121 |
| SEQ ID NO:116 light chain variable region | DIVMTQSHKF MSTSLGDRVS ITCKASQDVG AAVAWYQQKP GQSPKLLIYW ASTRHTGVPD<br>RFTGGGSGTD FTLTISNVQS EDLTDYFCQQ YINYPLTFGG GTKLEIKR | 60<br>108 |
| SEQ ID NO:117 heavy chain variable region of humanized antibody | QIQLVQSGPE LKKPGETVKI SCKASGYTFT DYSMHWVKQA PGKGLKWMGW INTETGEPTY<br>ADDFKGRFAF SLETSASTAY LQINNLKNED TATYFCANPY YDYVSYYAMD YWGHGTSVTV<br>SS | 60<br>120<br>122 |
| SEQ ID NO:118 heavy chain variable region of humanized antibody | QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY<br>ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV<br>SS | 60<br>120<br>122 |
| SEQ ID NO:119 light chain variable region of humanized antibody | DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD<br>RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK | 60<br>107 |
| SEQ ID NO:120 light chain variable region of humanized antibody | DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD<br>RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK | 60<br>107 |
| SEQ ID NO:121 heavy chain variable region of humanized antibody | EVQLVESGGG LVQPGESLKL SCESNEYEFP SHDMSWVRKT PEKRLELVAA INSDGGSTYY<br>PDTMERRFII SRDNTKKTLY LQMSSLRSED TALYYCARHY DDYYAWFAYW GQGTLVTVSA | 60<br>120 |
| SEQ ID NO:122 heavy chain variable region of humanized antibody | EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY<br>PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS | 60<br>120 |
| SEQ ID NO:123 light chain | DIVLTQSPAS LAVSLGQRAT ISCRASKSVS TSGYSYMHWY QQKPGQPPKL LIYLASNLES<br>GVPARFSGSG SGTDFTLNIH PVEEEDAATY YCQHSRELPL TFGAGTKLEL K | 60<br>111 |

| | | |
|---|---|---|
| variable region of humanized antibody | | |
| SEQ ID NO:124 light chain variable region of humanized antibody | EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES<br>GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K | 60<br>111 |
| SEQ ID NO:125 heavy chain variable region | MYLGLNYVFI VFLLNGVQSE VKLEESGGGL VQPGGSMKLS CAASGFTFSD AWMDWVRQSP<br>EKGLEWVAEI RSKANNHATY YAESVNGRFT ISRDDSKSSV YLQMNSLRAE DTGIYYCTWG<br>EVFYFDYWGQ GTTLTVSS | 60<br>120<br>138 |
| SEQ ID NO:126 light chain variable region | MRPSIQFLGL LLFWLHGAQC DIQMTQSPSS LSASLGGKVT ITCKSSQDIN KYIAWYQHKP<br>GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLLTFGAG<br>TKLELK | 60<br>120<br>126 |

**[00651]** In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain wherein each of the soluble OX40 binding domains lacks a stalk region (which contributes to trimerisation and provides a certain distance to the cell membrane, but is not part of the OX40 binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

**[00652]** In an embodiment, the OX40 agonist is an OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain, (iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein the TNF superfamily cytokine domain is an OX40 binding domain.

**[00653]** In some embodiments, the OX40 agonist is MEDI6383. MEDI6383 is an OX40 agonistic fusion protein and can be prepared as described in U.S. Patent No. 6,312,700, the disclosure of which is incorporated by reference herein.

**[00654]** In an embodiment, the OX40 agonist is an OX40 agonistic scFv antibody comprising any of the foregoing $V_H$ domains linked to any of the foregoing $V_L$ domains.

**[00655]** In an embodiment, the OX40 agonist is Creative Biolabs OX40 agonist monoclonal antibody MOM-18455, commercially available from Creative Biolabs, Inc., Shirley, NY, USA.

**[00656]** In an embodiment, the OX40 agonist is OX40 agonistic antibody clone Ber-ACT35 commercially available from BioLegend, Inc., San Diego, CA, USA.

**H.** Optional Cell Viability Analyses

**[00657]** Optionally, a cell viability assay can be performed after the first expansion (sometimes referred to as the initial bulk expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. Other assays for use in testing viability can include but are not limited to the Alamar blue assay; and the MTT assay.

1. Cell Counts, Viability, Flow Cytometry

**[00658]** In some embodiments, cell counts and/or viability are measured. The expression of markers such as but not limited CD3, CD4, CD8, and CD56, as well as any other disclosed or described herein, can be measured by flow cytometry with antibodies, for example but not limited to those commercially available from BD Bio-sciences (BD Biosciences, San Jose, CA) using a FACSCanto™ flow cytometer (BD Biosciences). The cells can be counted manually using a disposable c-chip hemocytometer (VWR, Batavia, IL) and viability can be assessed using any method known in the art, including but not limited to trypan blue staining. The cell viability can also be assayed based on USSN 15/863,634, incorporated by reference herein in its entirety.

**[00659]** In some cases, the bulk TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to REP and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the bulk or REP TIL populations can be subjected to genetic modifications for suitable treatments.

**[00660]** According to the present disclosure, a method for assaying TILs for viability and/or further use in administration to a subject. In some embodiments, the method for assay tumor infiltrating lymphocytes (TILs) comprises:

(i) obtaining a first population of TILs;

(ii) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a second population of TILs; and

(iii) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs;

(iv) harvesting, washing, and cryopreserving the third population of TILs;

(v) storing the cryopreserved TILs at a cryogenic temperature;

(vi) thawing the third population of TILs to provide a thawed third population of TILs; and

(vii) performing an additional second expansion of a portion of the thawed third population of TILs by supplementing the cell culture medium of the third population with IL-2, OKT-3, and APCs for an additional expansion period (sometimes referred to as a reREP period) of at least 3 days, wherein the third expansion is performed to obtain a fourth population of TILs, wherein the number of TILs in the fourth population of TILs is compared to the number of TILs in the third population of TILs to obtain a ratio;

(viii) determining based on the ratio in step (vii) whether the thawed population of TILs is suitable for administration to a patient;

(ix) administering a therapeutically effective dosage of the thawed third population of TILs to the patient when the ratio of the number of TILs in the fourth population of TILs to the number of TILs in the third population of TILs is determined to be greater than 5:1 in step (viii).

**[00661]** In some embodiments, the TILs are assayed for viability after step (vii).

**[00662]** The present disclosure also provides further methods for assaying TILs. In some embodiments, the disclosure provides a method for assaying TILs comprising:

(i) obtaining a portion of a first population of cryopreserved TILs;

(ii) thawing the portion of the first population of cryopreserved TILs;

(iii) performing a first expansion by culturing the portion of the first population of

TILs in a cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs) for an additional expansion period (sometimes referred to as a reREP period) of at least 3 days, to produce a second population of TILs, wherein the portion from the first population of TILs is compared to the second population of TILs to obtain a ratio of the number of TILs, wherein the ratio of the number of TILs in the second population of TILs to the number of TILs in the portion of the first population of TILs is greater than 5:1;

(iv) determining based on the ratio in step (iii) whether the first population of TILs is suitable for use in therapeutic administration to a patient;

(v) determining the first population of TILs is suitable for use in therapeutic administration when the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is determined to be greater than 5:1 in step (iv).

**[00663]** In some embodiments, the ratio of the number of TILs in the second population of TILs to the number of TILs in the portion of the first population of TILs is greater than 50:1.

**[00664]** In some embodiments, the method further comprises performing expansion of the entire first population of cryopreserved TILs from step (i) according to the methods as described in any of the embodiments provided herein.

**[00665]** In some embodiments, the method further comprises administering the entire first population of cryopreserved TILs from step (i) to the patient.

2. Cell Cultures

**[00666]** In an embodiment, a method for expanding TILs, including those discussed above as well as exemplified in Figure 1, may include using about 5,000 mL to about 25,000 mL of cell medium, about 5,000 mL to about 10,000 mL of cell medium, or about 5,800 mL to about 8,700 mL of cell medium. In some embodiments, the media is a serum free medium. In some embodiments, the media in the first expansion is serum free. In some embodiments, the media in the second expansion is serum free. . In some embodiments, the media in the first expansion and the second are both serum free. In an embodiment, expanding the number of TILs uses no more than one type of cell culture medium. Any suitable cell culture medium may be used, *e.g.*, AIM-V cell medium (L-glutamine, 50 μM streptomycin sulfate, and 10 μM gentamicin sulfate) cell culture medium (Invitrogen, Carlsbad CA). In this regard, the

inventive methods advantageously reduce the amount of medium and the number of types of medium required to expand the number of TIL. In an embodiment, expanding the number of TIL may comprise feeding the cells no more frequently than every third or fourth day. Expanding the number of cells in a gas permeable container simplifies the procedures necessary to expand the number of cells by reducing the feeding frequency necessary to expand the cells.

**[00667]** In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME).

**[00668]** In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable container containing cell medium therein; obtaining TILs from the tumor tissue sample; expanding the number of TILs in a second gas permeable container containing cell medium for a duration of about 7 to 14 days, *e.g.*, about 11 days. In some embodiments pre-REP is about 7 to 14 days, *e.g.*, about 11 days. In some embodiments, REP is about 7 to 14 days, *e.g.*, about 11 days.

**[00669]** In an embodiment, TILs are expanded in gas-permeable containers. Gas-permeable containers have been used to expand TILs using PBMCs using methods, compositions, and devices known in the art, including those described in U.S. Patent Application Publication No. 2005/0106717 A1, the disclosures of which are incorporated herein by reference. In an embodiment, TILs are expanded in gas-permeable bags. In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the Xuri Cell Expansion System W25 (GE Healthcare). In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the WAVE Bioreactor System, also known as the Xuri Cell Expansion System W5 (GE Healthcare). In an embodiment, the cell expansion system includes a gas permeable cell bag with a volume selected from the group consisting of about 100 mL, about 200 mL, about 300 mL, about 400 mL, about 500 mL, about 600 mL, about 700 mL, about 800 mL, about 900 mL, about 1 L, about 2 L, about 3 L, about 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, and about 10 L.

**[00670]** In an embodiment, TILs can be expanded in G-Rex flasks (commercially available from Wilson Wolf Manufacturing). Such embodiments allow for cell populations to expand from about $5x10^5$ cells/$cm^2$ to between $10x10^6$ and $30x10^6$ cells/$cm^2$. In an embodiment this is without feeding. In an embodiment, this is without feeding so long as medium resides at a height of about 10 cm in the G-Rex flask. In an embodiment this is without feeding but with the addition of one or more cytokines. In an embodiment, the cytokine can be added as a bolus without any need to mix the cytokine with the medium. Such containers, devices, and methods are known in the art and have been used to expand TILs, and include those described in U.S. Patent Application Publication No. US 2014/0377739A1, International Publication No. WO 2014/210036 A1, U.S. Patent Application Publication No. us 2013/0115617 A1, International Publication No. WO 2013/188427 A1, U.S. Patent Application Publication No. US 2011/0136228 A1, U.S. Patent No. US 8,809,050 B2, International publication No. WO 2011/072088 A2, U.S. Patent Application Publication No. US 2016/0208216 A1, U.S. Patent Application Publication No. US 2012/0244133 A1, International Publication No. WO 2012/129201 A1, U.S. Patent Application Publication No. US 2013/0102075 A1, U.S. Patent No. US 8,956,860 B2, International Publication No. WO 2013/173835 A1, U.S. Patent Application Publication No. US 2015/0175966 A1, the disclosures of which are incorporated herein by reference. Such processes are also described in Jin *et al.*, *J. Immunotherapy,* **2012**, 35:283-292.

**I.** Optional Genetic Engineering of TILs

**[00671]** In some embodiments, the TILs are optionally genetically engineered to include additional functionalities, including, but not limited to, a high-affinity T cell receptor (TCR), *e.g.*, a TCR targeted at a tumor-associated antigen such as MAGE-1, HER2, or NY-ESO-1, or a chimeric antigen receptor (CAR) which binds to a tumor-associated cell surface molecule (*e.g.*, mesothelin) or lineage-restricted cell surface molecule (*e.g.*, CD19).

**J. Optional Cryopreservation of TILs**

**[00672]** As discussed above, and exemplified in Steps A through E as provided in Figure 1, cryopreservation can occur at numerous points throughout the TIL expansion process. In some embodiments, the expanded population of TILs after the second expansion (as provided for example, according to Step D of Figure 1) can be cryopreserved. Cryopreservation can be generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in

solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986. In some embodiments, the TILs are cryopreserved in 5% DMSO. In some embodiments, the TILs are cryopreserved in cell culture media plus 5% DMSO. In some embodiments, the TILs are cryopreserved according to the methods provided in Examples F and G.

**[00673]** When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

### K. Closed Systems for TIL Manufacturing

**[00674]** The present invention provides for the use of closed systems during the TIL culturing process. Such closed systems allow for preventing and/or reducing microbial contamination, allow for the use of fewer flasks, and allow for cost reductions. In some embodiments, the closed system uses two containers.

**[00675]** Such closed systems are well-known in the art and can be found, for example, at http://www.fda.gov/cber/guidelines.htm and https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076779.htm.

**[00676]** Sterile connecting devices (STCDs) produce sterile welds between two pieces of compatible tubing. This procedure permits sterile connection of a variety of containers and tube diameters. In some embodiments, the closed systems include luer lock and heat sealed systems as described in for example, Example G. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in Example G is employed. In some embodiments, the TILs are formulated into a final product formulation container according to the method described in Example G, section “Final Formulation and Fill”.

**[00677]** In some embodiments, the closed system uses one container from the time the tumor fragments are obtained until the TILs are ready for administration to the patient or cryopreserving. In some embodiments when two containers are used, the first container is a

closed G-container and the population of TILs is centrifuged and transferred to an infusion bag without opening the first closed G-container. In some embodiments, when two containers are used, the infusion bag is a HypoThermosol-containing infusion bag. A closed system or closed TIL cell culture system is characterized in that once the tumor sample and/or tumor fragments have been added, the system is tightly sealed from the outside to form a closed environment free from the invasion of bacteria, fungi, and/or any other microbial contamination.

**[00678]** In some embodiments, the reduction in microbial contamination is between about 5% and about 100%. In some embodiments, the reduction in microbial contamination is between about 5% and about 95%. In some embodiments, the reduction in microbial contamination is between about 5% and about 90%. In some embodiments, the reduction in microbial contamination is between about 10% and about 90%. In some embodiments, the reduction in microbial contamination is between about 15% and about 85%. In some embodiments, the reduction in microbial contamination is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or about 100%.

**[00679]** The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

**[00680]** Moreover, pH, carbon dioxide partial pressure and oxygen partial pressure of the TIL cell culture environment each vary as the cells are cultured. Consequently, even though a medium appropriate for cell culture is circulated, the closed environment still needs to be constantly maintained as an optimal environment for TIL proliferation. To this end, it is desirable that the physical factors of pH, carbon dioxide partial pressure and oxygen partial pressure within the culture liquid of the closed environment be monitored by means of a sensor, the signal whereof is used to control a gas exchanger installed at the inlet of the culture environment, and the that gas partial pressure of the closed environment be adjusted in real time according to changes in the culture liquid so as to optimize the cell culture environment. In some embodiments, the present invention provides a closed cell culture system which incorporates at the inlet to the closed environment a gas exchanger equipped with a monitoring device which measures the pH, carbon dioxide partial pressure and oxygen partial pressure of the closed environment, and optimizes the cell culture environment by automatically adjusting gas concentrations based on signals from the monitoring device.

**[00681]** In some embodiments, the pressure within the closed environment is continuously or intermittently controlled. That is, the pressure in the closed environment can be varied by means of a pressure maintenance device for example, thus ensuring that the space is suitable for growth of TILs in a positive pressure state, or promoting exudation of fluid in a negative pressure state and thus promoting cell proliferation. By applying negative pressure intermittently, moreover, it is possible to uniformly and efficiently replace the circulating liquid in the closed environment by means of a temporary shrinkage in the volume of the closed environment.

**[00682]** In some embodiments, optimal culture components for proliferation of the TILs can be substituted or added, and including factors such as IL-2 and/or OKT3, as well as combination, can be added.

### L. Optional Cryopreservation of TILs

**[00683]** Either the bulk TIL population or the expanded population of TILs can be optionally cryopreserved. In some embodiments, cryopreservation occurs on therapeutic TIL population. In some embodiments, cryopreservation occurs on the TILs harvested after the second expansion. In some embodiments, cryopreservation occurs on the TILs in exemplary Step F of Figure 1. In some embodiments, the TILs are cryopreserved in the infusion bag. In some embodiments, the TILs are cryopreserved prior to placement in an infusion bag. In some embodiments, the TILs are cryopreserved and not placed in an infusion bag. In some embodiments, cryopreservation is performed using a cryopreservation medium. In some embodiments, the cryopreservation media contains dimethylsulfoxide (DMSO). This is generally accomplished by putting the TIL population into a freezing solution, e.g. 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See, Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986.

**[00684]** When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

**[00685]** In a preferred embodiment, a population of TILs is cryopreserved using CS10 cryopreservation media (CryoStor 10, BioLife Solutions). In a preferred embodiment, a population of TILs is cryopreserved using a cryopreservation media containing dimethylsulfoxide (DMSO). In a preferred embodiment, a population of TILs is cryopreserved using a 1:1 (vol:vol) ratio of CS10 and cell culture media. In a preferred embodiment, a population of TILs is cryopreserved using about a 1:1 (vol:vol) ratio of CS10 and cell culture media, further comprising additional IL-2.

**[00686]** As discussed above in Steps A through E, cryopreservation can occur at numerous points throughout the TIL expansion process. In some embodiments, the bulk TIL population after the first expansion according to Step B or the expanded population of TILs after the one or more second expansions according to Step D can be cryopreserved. Cryopreservation can be generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See Sadeghi, *et al.*, *Acta Oncologica* **2013,** *52,* 978-986.

**[00687]** When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

**[00688]** In some cases, the Step B TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to Step C and Step D and then cryopreserved after Step D. Similarly, in the case where genetically modified TILs will be used in therapy, the Step B or Step D TIL populations can be subjected to genetic modifications for suitable treatments.

## IV. TIL Manufacturing Processes (Embodiments of GEN3 Processes, optionally including Defined Media)

**[00689]** Without being limited to any particular theory, it is believed that the priming first expansion that primes an activation of T cells followed by the rapid second expansion that boosts the activation of T cells as described in the methods of the invention allows the preparation of expanded T cells that retain a “younger” phenotype, and as such the expanded T cells of the invention are expected to exhibit greater cytotoxicity against cancer cells than T

cells expanded by other methods. In particular, it is believed that an activation of T cells that is primed by exposure to an anti-CD3 antibody (e.g. OKT-3), IL-2 and optionally antigen-presenting cells (APCs) and then boosted by subsequent exposure to additional anti-CD-3 antibody (e.g. OKT-3), IL-2 and APCs as taught by the methods of the invention limits or avoids the maturation of T cells in culture, yielding a population of T cells with a less mature phenotype, which T cells are less exhausted by expansion in culture and exhibit greater cytotoxicity against cancer cells. In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (a) performing the rapid second expansion by culturing T cells in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer of the T cells in the small scale culture to a second container larger than the first container, e.g., a G-REX 500MCS container, and culturing the T cells from the small scale culture in a larger scale culture in the second container for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out of the culture by: (a) performing the rapid second expansion by culturing T cells in a first small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the T cells from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the T cells from first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing T cells in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the T cells from the small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container, e.g., G-REX 500MCS containers, wherein in each second container the portion of the T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing T cells in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 4 days, and then (b) effecting the transfer and apportioning of the T cells from the small scale

culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, e.g., G-REX 500MCS containers, wherein in each second container the portion of the T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 5 days.

**[00690]** In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion begins to decrease, abate, decay or subside.

**[00691]** In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by at or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100%.

**[00692]** In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by a percentage in the range of at or about 1% to 100%.

**[00693]** In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by a percentage in the range of at or about 1% to 10%, 10% to 20%, 20% to 30%, 30% to 40%, 40% to 50%, 50% to 60%, 60% to 70%, 70% to 80%, 80% to 90%, or 90% to 100%.

**[00694]** In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by at least at or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99%.

**[00695]** In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by up to at or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%.

**[00696]** In some embodiments, the decrease in the activation of T cells effected by the priming first expansion is determined by a reduction in the amount of interferon gamma released by the T cells in response to stimulation with antigen.

**[00697]** In some embodiments, the priming first expansion of T cells is performed during a period of up to at or about 7 days or about 8 days.

**[00698]** In some embodiments, the priming first expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days.

**[00699]** In some embodiments, the priming first expansion of T cells is performed during a period of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days.

**[00700]** In some embodiments, the rapid second expansion of T cells is performed during a period of up to at or about 11 days.

**[00701]** In some embodiments, the rapid second expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

**[00702]** In some embodiments, the rapid second expansion of T cells is performed during a period of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

**[00703]** In some embodiments, the priming first expansion of T cells is performed during a period of from at or about 1 day to at or about 7 days and the rapid second expansion of T cells is performed during a period of from at or about 1 day to at or about 11 days.

**[00704]** In some embodiments, the priming first expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days and the rapid second expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

**[00705]** In some embodiments, the priming first expansion of T cells is performed during a period of from at or about 1 day to at or about 8 days and the rapid second expansion of T cells is performed during a period of from at or about 1 day to at or about 9 days.

**[00706]** In some embodiments, the priming first expansion of T cells is performed during a period of 8 days and the rapid second expansion of T cells is performed during a period of 9 days.

**[00707]** In some embodiments, the priming first expansion of T cells is performed during a period of from at or about 1 day to at or about 7 days and the rapid second expansion of T cells is performed during a period of from at or about 1 day to at or about 9 days.

**[00708]** In some embodiments, the priming first expansion of T cells is performed during a period of 7 days and the rapid second expansion of T cells is performed during a period of 9 days.

**[00709]** In some embodiments, the T cells are tumor infiltrating lymphocytes (TILs).

**[00710]** In some embodiments, the T cells are marrow infiltrating lymphocytes (MILs).

**[00711]** In some embodiments, the T cells are peripheral blood lymphocytes (PBLs).

**[00712]** In some embodiments, the T cells are obtained from a donor suffering from a cancer.

**[00713]** In some embodiments, the T cells are TILs obtained from a tumor excised from a patient suffering from a cancer.

**[00714]** In some embodiments, the T cells are MILs obtained from bone marrow of a patient suffering from a hematologic malignancy.

**[00715]** In some embodiments, the T cells are PBLs obtained from peripheral blood mononuclear cells (PBMCs) from a donor. In some embodiments, the donor is suffering from a cancer. In some embodiments, the cancer is the cancer is selected from the group consisting of melanoma, ovarian cancer, endometrial cancer, thyroid cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the cancer is selected from the group consisting of melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodments, the donor is suffering from a tumor. In some embodiments, the tumor is a liquid tumor. In some embodiments, the tumor is a solid tumor. In some embodiments, the donor is suffering from a hematologic malignancy.

**[00716]** In certain aspects of the present disclosure, immune effector cells, e.g., T cells, can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as FICOLL separation. In one preferred aspect, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one aspect, the cells collected by apheresis may be washed to remove the plasma fraction and, optionally, to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. In one aspect, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL gradient or by counterflow centrifugal elutriation.

**[00717]** In some embodiments, the T cells are PBLs separated from whole blood or apheresis product enriched for lymphocytes from a donor. In some embodiments, the donor is suffering from a cancer. In some embodiments, the cancer is the cancer is selected from the group consisting of melanoma, ovarian cancer, endometrial cancer, thyroid cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the cancer is selected from the group consisting of melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodments, the donor is suffering from a tumor. In some embodiments, the tumor is a liquid tumor. In some embodiments, the tumor is a solid tumor. In some embodiments, the donor is suffering from a hematologic malignancy. In some embodiments, the PBLs are isolated from whole blood or apheresis product enriched for lymphocytes by using positive or negative selection methods, i.e., removing the PBLs using a marker(s), e.g., CD3+ CD45+, for T cell phenotype, or removing non-T cell phenotype cells, leaving PBLs. In other embodiments, the PBLs are isolated by gradient centrifugation. Upon isolation of PBLs from donor tissue, the priming first expansion of PBLs can be initiated by

seeding a suitable number of isolated PBLs (in some embodiments, approximately $1\times10^7$ PBLs) in the priming first expansion culture according to the priming first expansion step of any of the methods described herein.

**[00718]** An exemplary TIL process known as process 3 (also referred to herein as GEN3) containing some of these features is depicted in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), and some of the advantages of this embodiment of the present invention over process 2A are described in Figures 1, 2, 30, and 31 (in particular, *e.g.*, Figure 8B and/or Figure 8C). Two embodiments of process 3 are shown in Figures 1 and 30 (in particular, *e.g.*, Figure 8B and/or Figure 8C). Process 2A or Gen 2 is also described in U.S. Patent Publication No. 2018/0280436, incorporated by reference herein in its entirety. The Gen 3 process is also described in USSN 62/755,954 filed on November 5, 2018 (116983-5045-PR).

**[00719]** As discussed and generally outlined herein, TILs are taken from a patient sample and manipulated to expand their number prior to transplant into a patient using the TIL expansion process described herein and referred to as Gen 3. In some embodiments, the TILs may be optionally genetically manipulated as discussed below. In some embodiments, the TILs may be cryopreserved prior to or after expansion. Once thawed, they may also be restimulated to increase their metabolism prior to infusion into a patient.

**[00720]** In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) as Step B) is shortened to 1 to 8 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step D) is shortened to 1 to 9 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step B) is shortened to 1 to 8 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step D) is shortened to 1 to 8 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step B) is shortened to 1 to 7 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol

(REP) as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step D) is shortened to 1 to 9 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C) as Step B) is 1 to 7 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step D) is 1 to 10 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is shortened to 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 7 to 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 to 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is shortened to 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 7 to 8 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is shortened to 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 7 to 10 days. In some embodiments, the priming first

expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) is 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) is 8 to 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) is 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) is 9 to 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) is shortened to 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) is 7 to 9 days. In some embodiments, the combination of the priming first expansion and rapid second expansion (for example, expansions described as Step B and Step D in Figure 1 (in particular, *e.g.*, Figure 1B and/or Figure 8C)) is 14-16 days, as discussed in detail below and in the examples and figures. Particularly, it is considered that certain embodiments of the present invention comprise a priming first expansion step in which TILs are activated by exposure to an anti-CD3 antibody, *e.g.*, OKT-3 in the presence of IL-2 or exposure to an antigen in the presence of at least IL-2 and an anti-CD3 antibody *e.g.* OKT-3. In certain embodiments, the TILs which are activated in the priming first expansion step as described above are a first population of TILs *i.e.*, which are a primary cell population.

**[00721]** The "Step" Designations A, B, C, *etc.*, below are in reference to the non-limiting example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) and in reference to certain non-limiting embodiments described herein. The ordering of the Steps below and in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) is exemplary and any combination or order of steps, as well as additional steps, repetition of steps, and/or omission of steps is contemplated by the present application and the methods disclosed herein.

### A. STEP A: Obtain Patient tumor sample

**[00722]** In general, TILs are initially obtained from a patient tumor sample ("primary TILs") or from circulating lymphocytes, such as peripheral blood lymphocytes, including peripheral blood lymphocytes having TIL-like characteristics, and are then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, and optionally evaluated for phenotype and metabolic parameters as an indication of TIL health.

**[00723]** A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of any cancer type, including, but not limited to, breast, pancreatic, prostate, colorectal, lung, brain, renal, stomach, and skin (including but not limited to squamous cell carcinoma, basal cell carcinoma, and melanoma). In some embodiments, the cancer is selected from cervical cancer, head and neck cancer (including, for example, head and neck squamous cell carcinoma (HNSCC)), glioblastoma (GBM), gastrointestinal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, and non-small cell lung carcinoma. In some embodiments, useful TILs are obtained from malignant melanoma tumors, as these have been reported to have particularly high levels of TILs.

**[00724]** Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 $mm^3$, with from about 2-3 $mm^3$ being particularly useful. The TILs are cultured from these fragments using enzymatic tumor digests. Such tumor digests may be produced by incubation in enzymatic media (*e.g.*, Roswell Park Memorial Institute (RPMI) 1640 buffer, 2 mM glutamate, 10 mcg/mL gentamicine, 30 units/mL of DNase and 1.0 mg/mL of collagenase) followed by mechanical dissociation (*e.g.*, using a tissue dissociator). Tumor digests may be produced by placing the tumor in enzymatic media and mechanically dissociating the tumor for approximately 1 minute, followed by incubation for 30 minutes at 37 °C in 5% $CO_2$, followed by repeated cycles of mechanical dissociation and incubation under the foregoing conditions until only small tissue pieces are present. At the end of this process, if the cell suspension contains a large number of red blood cells or dead cells, a density gradient separation using FICOLL branched hydrophilic polysaccharide may be performed to remove these cells. Alternative methods known in the art may be used, such as those described in U.S. Patent Application Publication No. 2012/0244133 A1, the disclosure of which is incorporated by reference herein. Any of the foregoing methods may be used in any of the embodiments described herein for methods of expanding TILs or methods treating a cancer.

**[00725]** As indicated above, in some embodiments, the TILs are derived from solid tumors. In some embodiments, the solid tumors are not fragmented. In some embodiments, the solid

tumors are not fragmented and are subjected to enzymatic digestion as whole tumors. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% $CO_2$. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% $CO_2$ with rotation. In some embodiments, the tumors are digested overnight with constant rotation. In some embodiments, the tumors are digested overnight at 37°C, 5% $CO_2$ with constant rotation. In some embodiments, the whole tumor is combined with with the enzymes to form a tumor digest reaction mixture.

**[00726]** In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

**[00727]** In some embodiments, the enxyme mixture comprises collagenase. In some embodiments, the collagenase is collagenase IV. In some embodiments, the working stock for the collagenase is a 100 mg/ml 10X working stock.

**[00728]** In some embodiments, the enzyme mixture comprises DNAse. In some embodiments, the working stock for the DNAse is a 10,000IU/ml 10X working stock.

**[00729]** In some embodiments, the enzyme mixture comprises hyaluronidase. In some embodiments, the working stock for the hyaluronidase is a 10-mg/ml 10X working stock.

**[00730]** In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml DNAse, and 1 mg/ml hyaluronidase.

**[00731]** In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml DNAse, and 1 mg/ml hyaluronidase.

**[00732]** In general, the cell suspension obtained from the tumor is called a “primary cell population” or a “freshly obtained” or a “freshly isolated” cell population. In certain embodiments, the freshly obtained cell population of TILs is exposed to a cell culture medium comprising antigen presenting cells, IL-12 and OKT-3.

**[00733]** In some embodiments, fragmentation includes physical fragmentation, including, for example, dissection as well as digestion. In some embodiments, the fragmentation is physical fragmentation. In some embodiments, the fragmentation is dissection. In some

embodiments, the fragmentation is by digestion. In some embodiments, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients. In an embodiment, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients.

**[00734]** In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained in, for example, Step A (as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the step of fragmentation is an *in vitro* or *ex-vivo* process. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the priming first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the priming first expansion. In some embodiments, the tumor is fragmented and 40 fragments or pieces are placed in each container for the priming first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 $mm^3$. In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 $mm^3$ to about 1500 $mm^3$. In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 $mm^3$. In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams. In some embodiments, the multiple fragments comprise about 4 fragments.

**[00735]** In some embodiments, the TILs are obtained from tumor fragments. In some embodiments, the tumor fragment is obtained by sharp dissection. In some embodiments, the tumor fragment is between about 1 $mm^3$ and 10 $mm^3$. In some embodiments, the tumor fragment is between about 1 $mm^3$ and 8 $mm^3$. In some embodiments, the tumor fragment is about 1 $mm^3$. In some embodiments, the tumor fragment is about 2 $mm^3$. In some embodiments, the tumor fragment is about 3 $mm^3$. In some embodiments, the tumor fragment is about 4 $mm^3$. In some embodiments, the tumor fragment is about 5 $mm^3$. In some embodiments, the tumor fragment is about 6 $mm^3$. In some embodiments, the tumor fragment is about 7 $mm^3$. In some embodiments, the tumor fragment is about 8 $mm^3$. In some embodiments, the tumor fragment is about 9 $mm^3$. In some embodiments, the tumor fragment

is about 10 $mm^3$. In some embodiments, the tumor fragments are 1-4 mm x 1-4 mm x 1-4 mm. In some embodiments, the tumor fragments are 1 mm x 1 mm x 1 mm. In some embodiments, the tumor fragments are 2 mm x 2 mm x 2 mm. In some embodiments, the tumor fragments are 3 mm x 3 mm x 3 mm. In some embodiments, the tumor fragments are 4 mm x 4 mm x 4 mm.

**[00736]** In some embodiments, the tumors are fragmented in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of fatty tissue on each piece. In certain embodiments, the step of fragmentation of the tumor is an *in vitro* or *ex-vivo* method.

**[00737]** In some embodiments, the tumor fragmentation is performed in order to maintain the tumor internal structure. In some embodiments, the tumor fragmentation is performed without preforming a sawing motion with a scalpel. In some embodiments, the TILs are obtained from tumor digests. In some embodiments, tumor digests were generated by incubation in enzyme media, for example but not limited to RPMI 1640, 2 mM GlutaMAX, 10 mg/mL gentamicin, 30 U/mL DNase, and 1.0 mg/mL collagenase, followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). After placing the tumor in enzyme media, the tumor can be mechanically dissociated for approximately 1 minute. The solution can then be incubated for 30 minutes at 37 °C in 5% $CO_2$ and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% $CO_2$, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% $CO_2$. In some embodiments, at the end of the final incubation if the cell suspension contained a large number of red blood cells or dead cells, a density gradient separation using Ficoll can be performed to remove these cells.

**[00738]** In some embodiments, the cell suspension prior to the priming first expansion step is called a “primary cell population” or a “freshly obtained” or “freshly isolated” cell population.

**[00739]** In some embodiments, cells can be optionally frozen after sample isolation (*e.g.*, after obtaining the tumor sample and/or after obtaining the cell suspension from the tumor sample) and stored frozen prior to entry into the expansion described in Step B, which is described in further detail below, as well as exemplified in Figure 8 (in particular, *e.g.*, Figure 8B).

1. Core/Small Biopsy Derived TILS

**[00740]** In some embodiments, TILs are initially obtained from a patient tumor sample ("primary TILs") obtained by a core biopsy or similar procedure and then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, and optionally evaluated for phenotype and metabolic parameters.

**[00741]** In some emboidments, a patient tumor sample may be obtained using methods known in the art, generally via small biopsy, core biopsy, needle biopsy or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. In some embodiments, the sample can be from multiple small tumor samples or biopsies. In some embodiments, the sample can comprise multiple tumor samples from a single tumor from the same patient. In some embodiments, the sample can comprise multiple tumor samples from one, two, three, or four tumors from the same patient. In some embodiments, the sample can comprise multiple tumor samples from multiple tumors from the same patient. The solid tumor may of lung and/or non-small cell lung carcinoma (NSCLC).

**[00742]** In general, the cell suspension obtained from the tumor core or fragment is called a "primary cell population" or a "freshly obtained" or a "freshly isolated" cell population. In certain embodiments, the freshly obtained cell population of TILs is exposed to a cell culture medium comprising antigen presenting cells, IL-2 and OKT-3.

**[00743]** In some embodiments, if the tumor is metastatic and the primary lesion has been efficiently treated/removed in the past, removal of one of the metastatic lesions may be needed. In some embodiments, the least invasive approach is to remove a skin lesion, or a lymph node on the neck or axillary area when available. In some embodiments, a skin lesion is removed or small biopsy thereof is removed. In some embodiments, a lymph node or small

biopsy thereof is removed. In some embodiments, a lung or liver metastatic lesion, or an intra-abdominal or thoracic lymph node or small biopsy can thereof can be employed.

**[00744]** In some embodiments, the tumor is a melanoma. In some embodiments, the small biopsy for a melanoma comprises a mole or portion thereof.

**[00745]** In some embodiments, the small biopsy is a punch biopsy. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin. around a suspicious mole. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin, and a round piece of skin is removed. In some embodiments, the small biopsy is a punch biopsy and round portion of the tumor is removed.

**[00746]** In some embodiments, the small biopsy is an excisional biopsy. In some embodiments, the small biopsy is an excisional biopsy and the entire mole or growth is removed. In some embodiments, the small biopsy is an excisional biopsy and the entire mole or growth is removed along with a small border of normal-appearing skin.

**[00747]** In some embodiments, the small biopsy is an incisional biopsy. In some embodiments, the small biopsy is an incisional biopsy and only the most irregular part of a mole or growth is taken. In some embodiments, the small biopsy is an incisional biopsy and the incisional biopsy is used when other techniques can't be completed, such as if a suspicious mole is very large.

**[00748]** In some embodiments, the small biopsy is a lung biopsy. In some embodiments, the small biopsy is obtained by bronchoscopy. Generally, bronchoscopy, the patient is put under anesthesia, and a small tool goes through the nose or mouth, down the throat, and into the bronchial passages, where small tools are used to remove some tissue. In some embodiments, where the tumor or growth cannot be reached via bronchoscopy, a transthoracic needle biopsy can be employed. Generally, for a transthoracic needle biopsy, the patient is also under anesthesia and a needle is inserted through the skin directly into the suspicious spot to remove a small sample of tissue. In some embodiments, a transthoracic needle biopsy may require interventional radiology (for example, the use of x-rays or CT scan to guide the needle). In some embodiments, the small biopsy is obtained by needle biopsy. In some embodiments, the small biopsy is obtained endoscopic ultrasound (for example, an endoscope with a light and is placed through the mouth into the esophagus). In some embodiments, the small biopsy is obtained surgically.

**[00749]** In some embodiments, the small biopsy is a head and neck biopsy. In some embodiments, the small biopsy is an incisional biopsy. In some embodiments, the small biopsy is an incisional biopsy, wherein a small piece of tissue is cut from an abnormal-looking area. In some embodiments, if the abnormal region is easily accessed, the sample may be taken without hospitalization. In some embodiments, if the tumor is deeper inside the mouth or throat, the biopsy may need to be done in an operating room, with general anesthesia. In some embodiments, the small biopsy is an excisional biopsy. In some embodiments, the small biopsy is an excisional biopsy, wherein the whole area is removed. In some embodiments, the small biopsy is a fine needle aspiration (FNA). In some embodiments, the small biopsy is a fine needle aspiration (FNA), wherein a very thin needle attached to a syringe is used to extract (aspirate) cells from a tumor or lump. In some embodiments, the small biopsy is a punch biopsy. In some embodiments, the small biopsy is a punch biopsy, wherein punch forceps are used to remove a piece of the suspicious area.

**[00750]** In some embodiments, the small biopsy is a cervical biopsy. In some embodiments, the small biopsy is obtained via colposcopy. Generally, colposcopy methods employ the use of a lighted magnifying instrument attached to magnifying binoculars (a colposcope) which is then used to biopsy a small section of the surface of the cervix. In some embodiments, the small biopsy is a conization/cone biopsy. In some embodiments, the small biopsy is a conization/cone biopsy, wherein an outpatient surgery may be needed to remove a larger piece of tissue from the cervix. In some embodiments, the cone biopsy, in addition to helping to confirm a diagnosis, a cone biopsy can serve as an initial treatment.

**[00751]** The term “solid tumor” refers to an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors may be benign or malignant. The term “solid tumor cancer refers to malignant, neoplastic, or cancerous solid tumors. Solid tumor cancers include cancers of the lung. In some embodiments, the cancer is non-small cell lung carcinoma (NSCLC). The tissue structure of solid tumors includes interdependent tissue compartments including the parenchyma (cancer cells) and the supporting stromal cells in which the cancer cells are dispersed and which may provide a supporting microenvironment.

**[00752]** In some embodiments, the sample from the tumor is obtained as a fine needle aspirate (FNA), a core biopsy, a small biopsy (including, for example, a punch biopsy). In some embodiments, sample is placed first into a G-Rex 10. In some embodiments, sample is placed first into a G-Rex 10 when there are 1 or 2 core biopsy and/or small biopsy samples. In some embodiments, sample is placed first into a G-Rex 100 when there are 3, 4, 5, 6, 8, 9,

or 10 or more core biopsy and/or small biopsy samples. In some embodiments, sample is placed first into a G-Rex 500 when there are 3, 4, 5, 6, 8, 9, or 10 or more core biopsy and/or small biopsy samples.

**[00753]** The FNA can be obtained from a lung tumor, including, for example, an NSCLC. In some embodiments, the FNA is obtained from a lung tumor, such as a lung tumor from a patient with non-small cell lung cancer (NSCLC). In some cases, the patient with NSCLC has previously undergone a surgical treatment.

**[00754]** TILs described herein can be obtained from an FNA sample. In some cases, the FNA sample is obtained or isolated from the patient using a fine gauge needle ranging from an 18 gauge needle to a 25 gauge needle. The fine gauge needle can be 18 gauge, 19 gauge, 20 gauge, 21 gauge, 22 gauge, 23 gauge, 24 gauge, or 25 gauge. In some embodiments, the FNA sample from the patient can contain at least 400,000 TILs, *e.g.*, 400,000 TILs, 450,000 TILs, 500,000 TILs, 550,000 TILs, 600,000 TILs, 650,000 TILs, 700,000 TILs, 750,000 TILs, 800,000 TILs, 850,000 TILs, 900,000 TILs, 950,000 TILs, or more.

**[00755]** In some cases, the TILs described herein are obtained from a core biopsy sample. In some cases, the core biopsy sample is obtained or isolated from the patient using a surgical or medical needle ranging from an 11 gauge needle to a 16 gauge needle. The needle can be 11 gauge, 12 gauge, 13 gauge, 14 gauge, 15 gauge, or 16 gauge. In some embodiments, the core biopsy sample from the patient can contain at least 400,000 TILs, *e.g.*, 400,000 TILs, 450,000 TILs, 500,000 TILs, 550,000 TILs, 600,000 TILs, 650,000 TILs, 700,000 TILs, 750,000 TILs, 800,000 TILs, 850,000 TILs, 900,000 TILs, 950,000 TILs, or more.

**[00756]** In general, the harvested cell suspension is called a "primary cell population" or a "freshly harvested" cell population.

**[00757]** In some embodiments, the TILs are not obtained from tumor digests. In some embodiments, the solid tumor cores are not fragmented.

**[00758]** In some embodiments, the TILs are obtained from tumor digests. In some embodiments, tumor digests were generated by incubation in enzyme media, for example but not limited to RPMI 1640, 2mM GlutaMAX, 10 mg/mL gentamicin, 30 U/mL DNase, and 1.0 mg/mL collagenase, followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). After placing the tumor in enzyme media, the tumor can be mechanically dissociated for approximately 1 minute. The solution can then be incubated for 30 minutes at 37 °C in 5% $CO_2$ and it then mechanically disrupted again for approximately 1

minute. After being incubated again for 30 minutes at 37 °C in 5% $CO_2$, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% $CO_2$. In some embodiments, at the end of the final incubation if the cell suspension contained a large number of red blood cells or dead cells, a density gradient separation using Ficoll can be performed to remove these cells.

2. Methods of Expanding Peripheral Blood Lymphocytes (PBLs) from Peripheral Blood

**[00759]** PBL Method 1. In an embodiment of the invention, PBLs are expanded using the processes described herein. In an embodiment of the invention, the method comprises obtaining a PBMC sample from whole blood. In an embodiment, the method comprises enriching T-cells by isolating pure T-cells from PBMCs using negative selection of a non-CD19+ fraction. In an embodiment, the method comprises enriching T-cells by isolating pure T-cells from PBMCs using magnetic bead-based negative selection of a non-CD19+ fraction.

**[00760]** In an embodiment of the invention, PBL Method 1 is performed as follows: On Day 0, a cryopreserved PBMC sample is thawed and PBMCs are counted. T-cells are isolated using a Human Pan T-Cell Isolation Kit and LS columns (Miltenyi Biotec).

**[00761]** PBL Method 2. In an embodiment of the invention, PBLs are expanded using PBL Method 2, which comprises obtaining a PBMC sample from whole blood. The T-cells from the PBMCs are enriched by incubating the PBMCs for at least three hours at 37°C and then isolating the non-adherent cells.

**[00762]** In an embodiment of the invention, PBL Method 2 is performed as follows: On Day 0, the cryopreserved PMBC sample is thawed and the PBMC cells are seeded at 6 million cells per well in a 6 well plate in CM-2 media and incubated for 3 hours at 37 degrees Celsius. After 3 hours, the non-adherent cells, which are the PBLs, are removed and counted.

**[00763]** PBL Method 3. In an embodiment of the invention, PBLs are expanded using PBL Method 3, which comprises obtaining a PBMC sample from peripheral blood. B-cells are isolated using a CD19+ selection and T-cells are selected using negative selection of the non-CD19+ fraction of the PBMC sample.

**[00764]** In an embodiment of the invention, PBL Method 3 is performed as follows: On Day 0, cryopreserved PBMCs derived from peripheral blood are thawed and counted. CD19+ B-cells are sorted using a CD19 Multisort Kit, Human (Miltenyi Biotec). Of the non-CD19+ cell fraction, T-cells are purified using the Human Pan T-cell Isolation Kit and LS Columns (Miltenyi Biotec).

**[00765]** In an embodiment, PBMCs are isolated from a whole blood sample. In an embodiment, the PBMC sample is used as the starting material to expand the PBLs. In an embodiment, the sample is cryopreserved prior to the expansion process. In another embodiment, a fresh sample is used as the starting material to expand the PBLs. In an embodiment of the invention, T-cells are isolated from PBMCs using methods known in the art. In an embodiment, the T-cells are isolated using a Human Pan T-cell isolation kit and LS columns. In an embodiment of the invention, T-cells are isolated from PBMCs using antibody selection methods known in the art, for example, CD19 negative selection.

**[00766]** In an embodiment of the invention, the PBMC sample is incubated for a period of time at a desired temperature effective to identify the non-adherent cells. In an embodiment of the invention, the incubation time is about 3 hours. In an embodiment of the invention, the temperature is about 37° Celsius. The non-adherent cells are then expanded using the process described above.

**[00767]** In some embodiments, the PBMC sample is from a subject or patient who has been optionally pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor. In some embodiments, the tumor sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor. In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor, has undergone treatment for at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, or 1 year or more. In another embodiment, the PBMCs are derived from a patient who is currently on an ITK inhibitor regimen, such as ibrutinib.

**[00768]** In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor and is refractory to treatment with a kinase inhibitor or an ITK inhibitor, such as ibrutinib.

**[00769]** In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor but is no longer

undergoing treatment with a kinase inhibitor or an ITK inhibitor. In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor but is no longer undergoing treatment with a kinase inhibitor or an ITK inhibitor and has not undergone treatment for at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, or at least 1 year or more. In another embodiment, the PBMCs are derived from a patient who has prior exposure to an ITK inhibitor, but has not been treated in at least 3 months, at least 6 months, at least 9 months, or at least 1 year.

**[00770]** In an embodment of the invention, at Day 0, cells are selected for CD19+ and sorted accordingly. In an embodiment of the invention, the selection is made using antibody binding beads. In an embodiment of the invention, pure T-cells are isolated on Day 0 from the PBMCs.

**[00771]** In an embodiment of the invention, for patients that are not pre-treated with ibrutinib or other ITK inhibitor, 10-15ml of Buffy Coat will yield about $5\times10^9$ PBMC, which, in turn, will yield about $5.5\times10^7$ PBLs.

**[00772]** In an embodiment of the invention, for patients that are pre-treated with ibrutinib or other ITK inhibitor, the expansion process will yield about $20\times10^9$ PBLs. In an embodiment of the invention, $40.3\times10^6$ PBMCs will yield about $4.7\times10^5$ PBLs.

**[00773]** In any of the foregoing embodiments, PBMCs may be derived from a whole blood sample, by apheresis, from the buffy coat, or from any other method known in the art for obtaining PBMCs.

### 3. Methods of Expanding Marrow Infiltrating Lymphocytes (MILs) from PBMCs Derived from Bone Marrow

**[00774]** MIL Method 3. In an embodiment of the invention, the method comprises obtaining PBMCs from the bone marrow. On Day 0, the PBMCs are selected for CD3+/CD33+/CD20+/CD14+ and sorted, and the non-CD3+/CD33+/CD20+/CD14+ cell fraction is sonicated and a portion of the sonicated cell fraction is added back to the selected cell fraction.

**[00775]** In an embodiment of the invention, MIL Method 3 is performed as follows: On Day 0, a cryopreserved sample of PBMCs is thawed and PBMCs are counted. The cells are stained with CD3, CD33, CD20, and CD14 antibodies and sorted using a S3e cell sorted (Bio-Rad). The cells are sorted into two fractions – an immune cell fraction (or the MIL fraction) (CD3+CD33+CD20+CD14+) and an AML blast cell fraction (non-CD3+CD33+CD20+CD14+).

**[00776]** In an embodiment of the invention, PBMCs are obtained from bone marrow. In an embodiment, the PBMCs are obtained from the bone marrow through apheresis, aspiration, needle biopsy, or other similar means known in the art. In an embodiment, the PBMCs are fresh. In another embodiment, the PBMCs are cryopreserved.

**[00777]** In an embodiment of the invention, MILs are expanded from 10-50 ml of bone marrow aspirate. In an embodiment of the invention, 10ml of bone marrow aspirate is obtained from the patient. In another embodiment, 20ml of bone marrow aspirate is obtained from the patient. In another embodiment, 30ml of bone marrow aspirate is obtained from the patient. In another embodiment, 40ml of bone marrow aspirate is obtained from the patient. In another embodiment, 50ml of bone marrow aspirate is obtained from the patient.

**[00778]** In an embodiment of the invention, the number of PBMCs yielded from about 10-50ml of bone marrow aspirate is about $5\times10^7$ to about $10\times10^7$ PBMCs. In another embodiment, the number of PMBCs yielded is about $7\times10^7$ PBMCs.

**[00779]** In an embodiment of the invention, about $5\times10^7$ to about $10\times10^7$ PBMCs, yields about $0.5\times10^6$ to about $1.5\times10^6$ MILs. In an embodiment of the invention, about $1\times10^6$ MILs is yielded.

**[00780]** In an embodiment of the invention, $12\times10^6$ PBMC derived from bone marrow aspirate yields approximately $1.4\times10^5$ MILs.

**[00781]** In any of the foregoing embodiments, PBMCs may be derived from a whole blood sample, from bone marrow, by apheresis, from the buffy coat, or from any other method known in the art for obtaining PBMCs.

### B. STEP B: Priming First Expansion

**[00782]** In some embodiments, the present methods provide for younger TILs, which may provide additional therapeutic benefits over older TILs (*i.e.*, TILs which have further

undergone more rounds of replication prior to administration to a subject/patient). Features of young TILs have been described in the literature, for example Donia, at al., *Scandinavian Journal of Immunology,* 75:157–167 (2012); Dudley et al., *Clin Cancer Res,* 16:6122-6131 (2010); Huang et al., *J Immunother*, 28(3):258–267 (2005); Besser et al., *Clin Cancer Res*, 19(17):OF1-OF9 (2013); Besser et al., *J Immunother* 32:415–423 (2009); Robbins, et al., *J Immunol* 2004; 173:7125-7130; Shen et al., J Immunother, 30:123–129 (2007); Zhou, et al., *J Immunother*, 28:53–62 (2005); and Tran, et al., J Immunother, 31:742–751 (2008), all of which are incorporated herein by reference in their entireties.

**[00783]** After dissection or digestion of tumor fragments and/or tumor fragments, for example such as described in Step A of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), the resulting cells are cultured in serum containing IL-2, OKT-3, and feeder cells (*e.g.*, antigen-presenting feeder cells), under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the IL-2, OKT-3, and feeder cells are added at culture initiation along with the tumor digest and/or tumor fragments (*e.g.*, at Day 0). In some embodiments, the tumor digests and/or tumor fragments are incubated in a container with up to 60 fragments per container and with 6000 IU/mL of IL-2. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 8 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 7 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, priming first expansion occurs for a period of 1 to 8 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, priming first expansion occurs for a period of 1 to 7 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of 5 to 8 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of 5 to 7 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 6 to 8 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 6 to 7 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 7 to 8 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period

of about 7 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 8 days, resulting in a bulk TIL population, generally about $1 \times 10^8$ bulk TIL cells.

**[00784]** In a preferred embodiment, expansion of TILs may be performed using a priming first expansion step (for example such as those described in Step B of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), which can include processes referred to as pre-REP or priming REP and which contains feeder cells from Day 0 and/or from culture initiation) as described below and herein, followed by a rapid second expansion (Step D, including processes referred to as rapid expansion protocol (REP) steps) as described below under Step D and herein, followed by optional cryopreservation, and followed by a second Step D (including processes referred to as restimulation REP steps) as described below and herein. The TILs obtained from this process may be optionally characterized for phenotypic characteristics and metabolic parameters as described herein. In some embodiments, the tumor fragment is between about 1 $mm^3$ and 10 $mm^3$.

**[00785]** In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, CM for Step B consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin.

**[00786]** In some embodiments, there are less than or equal to 240 tumor fragments. In some embodiments, there are less than or equal to 240 tumor fragments placed in less than or equal to 4 containers. In some embodiments, the containers are GREX100 MCS flasks. In some embodiments, less than or equal to 60 tumor fragments are placed in 1 container. In some embodiments, each container comprises less than or equal to 500 mL of media per container. In some embodiments, the media comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media comprises antigen-presenting feeder cells (also referred to herein as "antigen-presenting cells"). In some embodiments, the media comprises $2.5 \times 10^8$ antigen-presenting feeder cells per container. In some embodiments, the media comprises OKT-3. In some embodiments, the media comprises 30 ng/mL of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng of OKT-3, and $2.5 \times 10^8$ antigen-presenting feeder cells. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and $2.5 \times 10^8$ antigen-presenting feeder cells per container.

**[00787]** After preparation of the tumor fragments, the resulting cells (*i.e.*, fragments which is a primary cell population) are cultured in media containing IL-2, antigen-presenting feeder cells and OKT-3 under conditions that favor the growth of TILs over tumor and other cells and which allow for TIL priming and accelerated growth from initiation of the culture on Day 0. In some embodiments, the tumor digests and/or tumor fragments are incubated in with 6000 IU/mL of IL-2, as well as antigen-presenting feeder cells and OKT-3. This primary cell population is cultured for a period of days, generally from 1 to 8 days, resulting in a bulk TIL population, generally about $1\times10^8$ bulk TIL cells. In some embodiments, the growth media during the priming first expansion comprises IL-2 or a variant thereof, as well as antigen-presenting feeder cells and OKT-3. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 7 days, resulting in a bulk TIL population, generally about $1\times10^8$ bulk TIL cells. In some embodiments, the growth media during the priming first expansion comprises IL-2 or a variant thereof, as well as antigen-presenting feeder cells and OKT-3. In some embodiments, the IL-2 is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of $20\text{-}30\times10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of $20\times10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of $25\times10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of $30\times10^6$ IU/mg for a 1 mg vial. In some embodiments, the IL- 2 stock solution has a final concentration of $4\text{-}8\times10^6$ IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of $5\text{-}7\times10^6$ IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of $6\times10^6$ IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepare as described in Example C. In some embodiments, the priming first expansion culture media comprises about 10,000 IU/mL of IL-2, about 9,000 IU/mL of IL-2, about 8,000 IU/mL of IL-2, about 7,000 IU/mL of IL-2, about 6000 IU/mL of IL-2 or about 5,000 IU/mL of IL-2. In some embodiments, the priming first expansion culture media comprises about 9,000 IU/mL of IL-2 to about 5,000 IU/mL of IL-2. In some embodiments, the priming first expansion culture media comprises about 8,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the priming first expansion culture media comprises about 7,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the priming first expansion culture media comprises about 6,000 IU/mL of IL-2. In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the priming first expansion cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the priming first expansion cell culture medium further comprises IL-2. In a

preferred embodiment, the priming first expansion cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the priming first expansion cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the priming first expansion cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or about 8000 IU/mL of IL-2.

**[00788]** In some embodiments, priming first expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the priming first expansion cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the priming first expansion cell culture medium further comprises IL-15. In a preferred embodiment, the priming first expansion cell culture medium comprises about 180 IU/mL of IL-15.

**[00789]** In some embodiments, priming first expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the priming first expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the priming first expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the priming first expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the priming first expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the priming first

expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the priming first expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the priming first expansion cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the priming first expansion cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the priming first expansion cell culture medium comprises about 1 IU/mL of IL-21.

**[00790]** In an embodiment, the priming first expansion cell culture medium comprises OKT-3 antibody. In some embodiments, the priming first expansion cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the priming first expansion cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 µg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 15 ng/ml and 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises 30 ng/mL of OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

TABLE 19: Amino acid sequences of muromonab (exemplary OKT-3 antibody)

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:1<br>Muromonab heavy chain | QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY<br>NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA<br>KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL<br>YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN<br>STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE<br>LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:2<br>Muromonab light chain | QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH<br>FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS<br>SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL<br>TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC | 60<br>120<br>180<br>213 |

**[00791]** In some embodiments, the priming first expansion cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 μg/mL and 100 μg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 μg/mL and 40 μg/mL.

**[00792]** In some embodiments, in addition to one or more TNFRSF agonists, the priming first expansion cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist. In some embodiments, in addition to one or more TNFRSF agonists, the priming first expansion cell culture medium further comprises IL-2 at an initial concentration of about 6000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

**[00793]** In some embodiments, the priming first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, it is referred to as CM1 (culture medium 1). In some embodiments, CM consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments, the CM is the CM1 described in the Examples, *see*, Example A. In some embodiments, the priming first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the priming first expansion culture medium or the initial cell culture medium or the first cell culture medium comprises IL-2, OKT-3 and antigen-presenting feeder cells (also referred to herein as feeder cells).

**[00794]** In some embodiments, the culture medium used in the expansion processes disclosed herein is a serum-free medium or a defined medium. In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or a serum replacement. In some embodiments, the serum-free or defined medium is used to prevent and/or decrease experimental variation due in part to the lot-to-lot variation of serum-containing media.

**[00795]** In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or serum replacement. In some embodiments, the basal cell medium includes, but is not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium , CTS™ OpTmizer™ T-Cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00796]** In some embodiments, the serum supplement or serum replacement includes, but is not limited to one or more of CTS™ OpTmizer T-Cell Expansion Serum Supplement, CTS™ Immune Cell Serum Replacement, one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more antibiotics, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties $Ag^{+}$, $Al^{3+}$, $Ba^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ge^{4+}$, $Se^{4+}$, Br, T, $Mn^{2+}$, P, $Si^{4+}$, $V^{5+}$, $Mo^{6+}$, $Ni^{2+}$, $Rb^{+}$, $Sn^{2+}$ and $Zr^{4+}$. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

**[00797]** In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00798]** In some embodiments, the total serum replacement concentration (vol%) in the serum-free or defined medium is from about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% by volume of the total serum-free

or defined medium. In some embodiments, the total serum replacement concentration is about 3% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 5% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 10% of the total volume of the serum-free or defined medium.

**[00799]** In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55μM.

**[00800]** In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR)

(ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55μM.

**[00801]** In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

**[00802]** In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about

150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55μM.

**[00803]** In some embodiments, the defined media described in International PCT Publication No. WO/1998/030679, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum- free culture. The serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties $Ag^{+}$, $Al^{3+}$, $Ba^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ge^{4+}$, $Se^{4+}$, Br, T, $Mn^{2+}$, P, $Si^{4+}$, $V^{5+}$, $Mo^{6+}$, $Ni^{2+}$, $Rb^{+}$, $Sn^{2+}$ and $Zr^{4+}$. In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00804]** In some embodiments, the concentration of glycine in the defined medium is in the range of from about 5-200 mg/L, the concentration of L- histidine is about 5-250 mg/L, the concentration of L-isoleucine is about 5-300 mg/L, the concentration of L-methionine is about 5-200 mg/L, the concentration of L-phenylalanine is about 5-400 mg/L, the concentration of L-proline is about 1-1000 mg/L, the concentration of L- hydroxyproline is about 1-45 mg/L, the concentration of L-serine is about 1-250 mg/L, the concentration of L-threonine is about 10-500 mg/L, the concentration of L-tryptophan is about 2-110 mg/L, the concentration of L-tyrosine is about 3-175 mg/L, the concentration of L-valine is about 5-500 mg/L, the concentration of thiamine is about 1-20 mg/L, the concentration of reduced glutathione is about 1-20 mg/L, the concentration of L-ascorbic acid-2-phosphate is about 1-200 mg/L, the concentration of iron saturated transferrin is about 1-50 mg/L, the concentration of insulin is about 1-100 mg/L, the concentration of sodium selenite is about 0.000001-0.0001 mg/L, and the concentration of albumin (e.g., AlbuMAX® I) is about 5000-50,000 mg/L.

**[00805]** In some embodiments, the non-trace element moiety ingredients in the defined medium are present in the concentration ranges listed in the column under the heading "Concentration Range in 1X Medium" in Table A below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading "A Preferred Embodiment of the 1X Medium" in Table A below. In other embodiments, the defined medium is a basal cell medium comprising a serum free supplement. In some of these embodiments, the serum free supplement comprises non-trace moiety ingredients of the type and in the concentrations listed in the column under the heading "A Preferred Embodiment in Supplement" in Table A below.

Table A: Concentrations of Non-Trace Element Moiety Ingredients

| Ingredient | A preferred embodiment in supplement (mg/L) (About) | Concentration range in 1X medium (mg/L) (About) | A preferred embodiment in 1X medium (mg/L) (About) |
|---|---|---|---|
| Glycine | 150 | 5-200 | 53 |
| L-Histidine | 940 | 5-250 | 183 |
| L-Isoleucine | 3400 | 5-300 | 615 |
| L-Methionine | 90 | 5-200 | 44 |
| L-Phenylalanine | 1800 | 5-400 | 336 |
| L-Proline | 4000 | 1-1000 | 600 |
| L-Hydroxyproline | 100 | 1-45 | 15 |

| | | | |
|---|---|---|---|
| L-Serine | 800 | 1-250 | 162 |
| L-Threonine | 2200 | 10-500 | 425 |
| L-Tryptophan | 440 | 2-110 | 82 |
| L-Tyrosine | 77 | 3-175 | 84 |
| L-Valine | 2400 | 5-500 | 454 |
| Thiamine | 33 | 1-20 | 9 |
| Reduced Glutathione | 10 | 1-20 | 1.5 |
| Ascorbic Acid-2-$PO_4$ (Mg Salt) | 330 | 1-200 | 50 |
| Transferrin (iron saturated) | 55 | 1-50 | 8 |
| Insulin | 100 | 1-100 | 10 |
| Sodium Selenite | 0.07 | 0.000001-0.0001 | 0.00001 |
| AlbuMAX®I | 83,000 | 5000-50,000 | 12,500 |

**[00806]** In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μM), 2-mercaptoethanol (final concentration of about 100 μM).

**[00807]** In some embodiments, the defined media described in Smith, *et al.*, "Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement," *Clin Transl Immunology*, 4(1) 2015 (doi: 10.1038/cti.2014.31) are useful in the present invention. Briefly, RPMI or CTS™ OpTmizer™ was used as the basal cell medium, and supplemented with either 0, 2%, 5%, or 10% CTS™ Immune Cell Serum Replacement.

**[00808]** In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME or βME; also known as 2-mercaptoethanol, CAS 60-24-2).

**[00809]** In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 1 to 8 days, as discussed in the examples and figures. In some embodiments, the priming

first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 2 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 3 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 4 to 8 days, as discussed in the examples and figures. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 1 to 7 days, as discussed in the examples and figures. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 2 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 2 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 3 to 8 days.In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 3 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 4 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 4 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process

is 5 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 5 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 6 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 6 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those provided in Step B of Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 7 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those provided in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 8 days.In some embodiments, the priming first expansion (including processes such as for example those provided in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 7 days.

**[00810]** In some embodiments, the priming first TIL expansion can proceed for 1 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 1 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 2 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 2 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 3 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 3 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 4 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is

initiated. In some embodiments, the priming first TIL expansion can proceed for 4 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 5 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 5 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 6 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated.In some embodiments, the priming first TIL expansion can proceed for 6 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 7 to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated.In some embodiments, the priming first TIL expansion can proceed for 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated.

**[00811]** In some embodiments, the priming first expansion of the TILs can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days. In some embodiments, the first TIL expansion can proceed for 1 day to 8 days. In some embodiments, the first TIL expansion can proceed for 1 day to 7 days. In some embodiments, the first TIL expansion can proceed for 2 days to 8 days. In some embodiments, the first TIL expansion can proceed for 2 days to 7 days. In some embodiments, the first TIL expansion can proceed for 3 days to 8 days. In some embodiments, the first TIL expansion can proceed for 3 days to 7 days. In some embodiments, the first TIL expansion can proceed for 4 days to 8 days. In some embodiments, the first TIL expansion can proceed for 4 days to 7 days. In some embodiments, the first TIL expansion can proceed for 5 days to 8 days. In some embodiments, the first TIL expansion can proceed for 5 days to 7 days. In some embodiments, the first TIL expansion can proceed for 6 days to 8 days. In some embodiments, the first TIL expansion can proceed for 6 days to 7 days. In some embodiments, the first TIL expansion can proceed for 7 to 8 days. In some embodiments, the first TIL expansion can proceed for 8 days. In some embodiments, the first TIL expansion can proceed for 7 days.

**[00812]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the priming first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the priming first expansion, including, for example during Step B processes according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the priming first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) and as described herein.

**[00813]** In some embodiments, the priming first expansion, for example, Step B according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-10.

1. Feeder Cells and Antigen Presenting Cells

**[00814]** In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 4-8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time

during days 4-7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 5-8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 5-7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 6-8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 6-7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during day 7 or 8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during day 7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP)

does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during day 8

**[00815]** In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.*, Figure 8B), as well as those referred to as pre-REP or priming REP) require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion and during the priming first expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, $2.5 \times 10^8$ feeder cells are used during the priming first expansion. In some embodiments, $2.5 \times 10^8$ feeder cells per container are used during the priming first expansion. In some embodiments, $2.5 \times 10^8$ feeder cells per GREX-10 are used during the priming first expansion. In some embodiments, $2.5 \times 10^8$ feeder cells per GREX-100 are used during the priming first expansion.

**[00816]** In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

**[00817]** In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the priming first expansion.

**[00818]** In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 have not increased from the initial viable cell number put into culture on day 0 of the priming first expansion. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 3000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 6000 IU/ml IL-2.

**[00819]** In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of

viable cells, cultured in the presence of OKT3 and IL-2, on day 7 have not increased from the initial viable cell number put into culture on day 0 of the priming first expansion. In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/mL OKT3 antibody and 1000-6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/ml OKT3 antibody and 2000-5000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/mL OKT3 antibody and 2000-4000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/mL OKT3 antibody and 2500-3500 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 15 ng/mL OKT3 antibody and 3000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 15 ng/mL OKT3 antibody and 6000 IU/ml IL-2.

**[00820]** In some embodiments, the antigen-presenting feeder cells are PBMCs. In some embodiments, the antigen-presenting feeder cells are artificial antigen-presenting feeder cells. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

**[00821]** In an embodiment, the priming first expansion procedures described herein require a ratio of about $2.5 \times 10^8$ feeder cells to about $100 \times 10^6$ TILs. In another embodiment, the priming first expansion procedures described herein require a ratio of about $2.5 \times 10^8$ feeder cells to about $50 \times 10^6$ TILs. In yet another embodiment, the priming first expansion described herein require about $2.5 \times 10^8$ feeder cells to about $25 \times 10^6$ TILs. In yet another embodiment, the priming first expansion described herein require about $2.5 \times 10^8$ feeder cells. In yet another embodiment, the priming first expansion requires one-fourth, one-third, five-twelfths, or one-half of the number of feeder cells used in the rapid second expansion.

**[00822]** In some embodiments, the media in the priming first expansion comprises IL-2. In some embodiments, the media in the priming first expansion comprises 6000 IU/mL of IL-2. In some embodiments, the media in the priming first expansion comprises antigen-presenting feeder cells. In some embodiments, the media in the priming first expansion comprises 2.5 ×

$10^8$ antigen-presenting feeder cells per container. In some embodiments, the media in the priming first expansion comprises OKT-3. In some embodiments, the media comprises 30 ng of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5 × $10^8$ antigen-presenting feeder cells. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5 × $10^8$ antigen-presenting feeder cells per container. In some embodiments, the media comprises 500 mL of culture medium and 15 µg of OKT-3 per 2.5 × $10^8$ antigen-presenting feeder cells per container. In some embodiments, the media comprises 500 mL of culture medium and 15 µg of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 500 mL of culture medium, 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5 × $10^8$ antigen-presenting feeder cells. In some embodiments, the media comprises 500 mL of culture medium, 6000 IU/mL of IL-2, 15 µg of OKT-3, and 2.5 × $10^8$ antigen-presenting feeder cells per container. In some embodiments, the media comprises 500 mL of culture medium and 15 µg of OKT-3 per 2.5 × $10^8$ antigen-presenting feeder cells per container.

**[00823]** In an embodiment, the priming first expansion procedures described herein require an excess of feeder cells over TILs during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In an embodiment, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

**[00824]** In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

**[00825]** In an embodiment, artificial antigen presenting cells are used in the priming first expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

**[00826]** The expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

**[00827]** Alternatively, using combinations of cytokines for the priming first expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in International Publication No. WO 2015/189356 and WO

2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21, and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

TABLE 20: Amino acid sequences of interleukins.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:3<br>recombinant<br>human IL-2<br>(rhIL-2) | MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL<br>EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN<br>RWITFCQSII STLT | 60<br>120<br>134 |
| SEQ ID NO:4<br>Aldesleukin | PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE<br>ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW<br>ITFSQSIIST LT | 60<br>120<br>132 |
| SEQ ID NO:5<br>recombinant<br>human IL-4<br>(rhIL-4) | MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH<br>EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI<br>MREKYSKCSS | 60<br>120<br>130 |
| SEQ ID NO:6<br>recombinant<br>human IL-7<br>(rhIL-7) | MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA<br>ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL<br>KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH | 60<br>120<br>153 |
| SEQ ID NO:7<br>recombinant<br>human IL-15<br>(rhIL-15) | MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI<br>HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS | 60<br>115 |
| SEQ ID NO:8<br>recombinant<br>human IL-21<br>(rhIL-21) | MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG<br>NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ<br>HLSSRTHGSE DS | 60<br>120<br>132 |

### C. STEP C: Priming First Expansion to Rapid Second Expansion Transition

**[00828]** In some cases, the bulk TIL population obtained from the priming first expansion (which can include expansions sometimes referred to as pre-REP), including, for example the TIL population obtained from for example, Step B as indicated in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), can be subjected to a rapid second expansion (which can include expansions sometimes referred to as Rapid Expansion Protocol (REP)) and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the expanded TIL population from the priming first expansion or the expanded TIL population from the rapid second expansion can be subjected to genetic modifications for suitable treatments prior to the expansion step or after the priming first expansion and prior to the rapid second expansion.

**[00829]** In some embodiments, the TILs obtained from the priming first expansion (for example, from Step B as indicated in Figure 1 (in particular, *e.g.*, Figure 1B and/or Figure

8C)) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the priming first expansion (for example, from Step B as indicated in Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C)) are not stored and proceed directly to the rapid second expansion. In some embodiments, the TILs obtained from the priming first expansion are not cryopreserved after the priming first expansion and prior to the rapid second expansion. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, or 8 days from when tumor fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs at about 3 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs at about 3 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 4 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 4 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 5 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 5 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 6 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 6 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 7 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion

occurs at about 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated.

**[00830]** In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs at 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 1 day to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 1 day to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 2 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 2 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 3 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 3 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 4 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 4 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 5 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 5 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 6 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. . In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 6 days to 8 days from when fragmentation occurs and/or when the

first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 7 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated

**[00831]** In some embodiments, the TILs are not stored after the primary first expansion and prior to the rapid second expansion, and the TILs proceed directly to the rapid second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)). In some embodiments, the transition occurs in closed system, as described herein. In some embodiments, the TILs from the priming first expansion, the second population of TILs, proceeds directly into the rapid second expansion with no transition period.

**[00832]** In some embodiments, the transition from the priming first expansion to the rapid second expansion, for example, Step C according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a GREX-10 or a GREX-100. In some embodiments, the closed system bioreactor is a single bioreactor. In some embodiments, the transition from the priming first expansion to the rapid second expansion involves a scale-up in container size. In some embodiments, the priming first expansion is performed in a smaller container than the rapid second expansion. In some embodiments, the priming first expansion is performed in a GREX-100 and the rapid second expansion is performed in a GREX-500.

### D. STEP D: Rapid Second Expansion

**[00833]** In some embodiments, the TIL cell population is further expanded in number after harvest and the priming first expansion, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)). This further expansion is referred to herein as the rapid second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (Rapid

Expansion Protocol or REP; as well as processes as indicated in Step D of Figure 8 (in particular, *e.g.*, Figure 8B)). The rapid second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container. In some embodiments, 1 day, 2 days, 3 days, or 4 days after initiation of the rapid second expansion (*i.e.*, at days 8, 9, 10, or 11 of the overall Gen 3 process), the TILs are transferred to a larger volume container.

**[00834]** In some embodiments, the rapid second expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 1 (in particular, *e.g.*, Figure 1B and/or Figure 8C)) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 1 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 1 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 2 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 2 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 3 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 3 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 4 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 4 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 5 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 5 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days to about 10 days

after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 9 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 1 day after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 2 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 3 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 4 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 5 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 10 days after initiation of the rapid second expansion.

**[00835]** In an embodiment, the rapid second expansion can be performed in a gas permeable container using the methods of the present disclosure (including, for example, expansions referred to as REP; as well as processes as indicated in Step D of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, the TILs are expanded in the rapid second expansion in the presence of IL-2, OKT-3, and feeder cells (also referred herein as "antigen-presenting cells"). In some embodiments, the TILs are expanded in the rapid second expansion in the presence of IL-2, OKT-3, and feeder cells, wherein the feeder cells are added to a final concentration that is twice, 2.4 times, 2.5 times, 3 times, 3.5 times or 4 times the concentration of feeder cells present in the priming first expansion. For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San

Diego, CA, USA). TILs can be expanded to induce further stimulation of the TILs *in vitro* by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 μM MART-1 :26-35 (27 L) or gpl 00:209-217 (210M), optionally in the presence of a T-cell growth factor, such as 300 IU/mL IL-2 or IL-15. Other suitable antigens may include, *e.g.*, NY-ESO-1, TRP-1, TRP-2, tyrosinase cancer antigen, MAGE-A3, SSX-2, and VEGFR2, or antigenic portions thereof. TIL may also be rapidly expanded by re-stimulation with the same antigen(s) of the cancer pulsed onto HLA-A2-expressing antigen-presenting cells. Alternatively, the TILs can be further re-stimulated with, *e.g.*, example, irradiated, autologous lymphocytes or with irradiated HLA-A2+ allogeneic lymphocytes and IL-2. In some embodiments, the re-stimulation occurs as part of the second expansion. In some embodiments, the second expansion occurs in the presence of irradiated, autologous lymphocytes or with irradiated HLA-A2+ allogeneic lymphocytes and IL-2.

**[00836]** In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or between 8000 IU/mL of IL-2.

**[00837]** In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 μg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL,

between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 15 ng/ml and 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 30 ng/ml and 60 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 30 ng/mL OKT-3. In an embodiment, the cell culture medium comprises about 60 ng/mL OKT-3. In some embodiments, the OKT-3 antibody is muromonab.

**[00838]** In some embodiments, the media in the rapid second expansion comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media in the rapid second expansion comprises antigen-presenting feeder cells. In some embodiments, the media in the rapid second expansion comprises $7.5 \times 10^8$ antigen-presenting feeder cells per container. In some embodiments, the media in the rapid second expansion comprises OKT-3. In some embodiments, the in the rapid second expansion media comprises 500 mL of culture medium and 30 μg of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the in the rapid second expansion media comprises 6000 IU/mL of IL-2, 60 ng/mL of OKT-3, and $7.5 \times 10^8$ antigen-presenting feeder cells. In some embodiments, the media comprises 500 mL of culture medium and 6000 IU/mL of IL-2, 30 μg of OKT-3, and $7.5 \times 10^8$ antigen-presenting feeder cells per container.

**[00839]** In some embodiments, the media in the rapid second expansion comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media in the rapid second expansion comprises antigen-presenting feeder cells. In some embodiments, the media comprises between $5 \times 10^8$ and $7.5 \times 10^8$ antigen-presenting feeder cells per container. In some embodiments, the media in the rapid second expansion comprises OKT-3. In some embodiments, the media in the rapid second expansion comprises 500 mL of culture medium and 30 μg of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media in the rapid second expansion comprises 6000 IU/mL of IL-2, 60 ng/mL of OKT-3, and between $5 \times 10^8$ and $7.5 \times 10^8$ antigen-presenting feeder cells. In some embodiments, the media in the rapid second expansion comprises 500 mL of culture medium and 6000 IU/mL of IL-2, 30 μg of OKT-3, and between $5 \times 10^8$ and $7.5 \times 10^8$ antigen-presenting feeder cells per container.

**[00840]** In some embodiments, the cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-

1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 μg/mL and 100 μg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 μg/mL and 40 μg/mL.

**[00841]** In some embodiments, in addition to one or more TNFRSF agonists, the cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

**[00842]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the second expansion, including, for example during a Step D processes according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step D processes according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) and as described herein.

**[00843]** In some embodiments, the second expansion can be conducted in a supplemented cell culture medium comprising IL-2, OKT-3, antigen-presenting feeder cells, and optionally a TNFRSF agonist. In some embodiments, the second expansion occurs in a supplemented cell culture medium. In some embodiments, the supplemented cell culture medium comprises IL-2, OKT-3, and antigen-presenting feeder cells. In some embodiments, the second cell culture medium comprises IL-2, OKT-3, and antigen-presenting cells (APCs; also referred to as antigen-presenting feeder cells). In some embodiments, the second expansion occurs in a cell culture medium comprising IL-2, OKT-3, and antigen-presenting feeder cells (*i.e.*, antigen presenting cells).

**[00844]** In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15,

about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the cell culture medium further comprises IL-15. In a preferred embodiment, the cell culture medium comprises about 180 IU/mL of IL-15.

**[00845]** In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the cell culture medium comprises about 1 IU/mL of IL-21.

**[00846]** In some embodiments, the antigen-presenting feeder cells (APCs) are PBMCs. In an embodiment, the ratio of TILs to PBMCs and/or antigen-presenting cells in the rapid expansion and/or the second expansion is about 1 to 10, about 1 to 15, about 1 to 20, about 1 to 25, about 1 to 30, about 1 to 35, about 1 to 40, about 1 to 45, about 1 to 50, about 1 to 75, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 50 and 1 to 300. In an embodiment,

the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 100 and 1 to 200.

**[00847]** In an embodiment, REP and/or the rapid second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, wherein the feeder cell concentration is at least 1.1 times (1.1X), 1.2X, 1.3X, 1.4X, 1.5X, 1.6X, 1.7X, 1.8X, 1.8X, 2X, 2.1X2.2X, 2.3X, 2.4X, 2.5X, 2.6X, 2.7X, 2.8X, 2.9X, 3.0X, 3.1X, 3.2X, 3.3X, 3.4X, 3.5X, 3.6X, 3.7X, 3.8X, 3.9X or 4.0X the feeder cell concentration in the priming first expansion, 30 ng/mL OKT3 anti-CD3 antibody and 6000 IU/mL IL-2 in 150 ml media. Media replacement is done (generally 2/3 media replacement via aspiration of 2/3 of spent media and replacement with an equal volume of fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[00848]** In some embodiments, the rapid second expansion (which can include processes referred to as the REP process) is 7 to 9 days, as discussed in the examples and figures. In some embodiments, the second expansion is 7 days. In some embodiments, the second expansion is 8 days. In some embodiments, the second expansion is 9 days.

**[00849]** In an embodiment, the second expansion (which can include expansions referred to as REP, as well as those referred to in Step D of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) may be performed in 500 mL capacity gas permeable flasks with 100 cm gas-permeable silicon bottoms (G-Rex 100, commercially available from Wilson Wolf Manufacturing Corporation, New Brighton, MN, USA), $5 \times 10^6$ or $10 \times 10^6$ TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per ml of anti-CD3 (OKT3). The G-Rex 100 flasks may be incubated at 37°C in 5% $CO_2$. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and centrifuged at 1500 rpm (491 × g) for 10 minutes. The TIL pellets may be re-suspended with 150 mL of fresh medium with 5% human AB serum, 6000 IU per mL of IL-2, and added back to the original GREX-100 flasks. When TIL are expanded serially in GREX-100 flasks, on day 10 or 11 the TILs can be moved to a larger flask, such as a GREX-500. The cells may be harvested on day 14 of culture. The cells may be harvested on day 15 of culture. The cells may be harvested on day 16 of culture. In some embodiments, media replacement is done until the cells are transferred to an alternative growth chamber. In some embodiments, 2/3 of the media is replaced by aspiration of spent media and

replacement with an equal volume of fresh media. In some embodiments, alternative growth chambers include GREX flasks and gas permeable containers as more fully discussed below.

[00850] In some embodiments, the culture medium used in the expansion processes disclosed herein is a serum-free medium or a defined medium. In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or a serum replacement. In some embodiments, the serum-free or defined medium is used to prevent and/or decrease experimental variation due in part to the lot-to-lot variation of serum-containing media.

[00851] In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or serum replacement. In some embodiments, the basal cell medium includes, but is not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium , CTS™ OpTmizer™ T-Cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00852]** In some embodiments, the serum supplement or serum replacement includes, but is not limited to one or more of CTS™ OpTmizer T-Cell Expansion Serum Supplement, CTS™ Immune Cell Serum Replacement, one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more antibiotics, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties $Ag^{+}$, $Al^{3+}$, $Ba^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ge^{4+}$, $Se^{4+}$, Br, T, $Mn^{2+}$, P, $Si^{4+}$, $V^{5+}$, $Mo^{6+}$, $Ni^{2+}$, $Rb^{+}$, $Sn^{2+}$ and $Zr^{4+}$. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

**[00853]** In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™

OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00854]** In some embodiments, the total serum replacement concentration (vol%) in the serum-free or defined medium is from about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% by volume of the total serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 3% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 5% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 10% of the total volume of the serum-free or defined medium.

**[00855]** In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM.

**[00856]** In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3%

of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 6000 IU/mL of IL-2.

**[00857]** In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

**[00858]** In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM.

**[00859]** In some embodiments, the defined media described in International PCT Publication No. WO/1998/030679, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum- free culture. The serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties $Ag^{+}$, $Al^{3+}$, $Ba^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Ge^{4+}$, $Se^{4+}$, Br, T, $Mn^{2+}$, P, $Si^{4+}$, $V^{5+}$, $Mo^{6+}$, $Ni^{2+}$, $Rb^{+}$, $Sn^{2+}$ and $Zr^{4+}$. In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM),

Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00860]** In some embodiments, the concentration of glycine in the defined medium is in the range of from about 5-200 mg/L, the concentration of L- histidine is about 5-250 mg/L, the concentration of L-isoleucine is about 5-300 mg/L, the concentration of L-methionine is about 5-200 mg/L, the concentration of L-phenylalanine is about 5-400 mg/L, the concentration of L-proline is about 1-1000 mg/L, the concentration of L- hydroxyproline is about 1-45 mg/L, the concentration of L-serine is about 1-250 mg/L, the concentration of L-threonine is about 10-500 mg/L, the concentration of L-tryptophan is about 2-110 mg/L, the concentration of L-tyrosine is about 3-175 mg/L, the concentration of L-valine is about 5-500 mg/L, the concentration of thiamine is about 1-20 mg/L, the concentration of reduced glutathione is about 1-20 mg/L, the concentration of L-ascorbic acid-2-phosphate is about 1-200 mg/L, the concentration of iron saturated transferrin is about 1-50 mg/L, the concentration of insulin is about 1-100 mg/L, the concentration of sodium selenite is about 0.000001-0.0001 mg/L, and the concentration of albumin (e.g., AlbuMAX® I) is about 5000-50,000 mg/L.

**[00861]** In some embodiments, the non-trace element moiety ingredients in the defined medium are present in the concentration ranges listed in the column under the heading "Concentration Range in 1X Medium" in Table A below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading "A Preferred Embodiment of the 1X Medium" in Table A below. In other embodiments, the defined medium is a basal cell medium comprising a serum free supplement. In some of these embodiments, the serum free supplement comprises non-trace moiety ingredients of the type and in the concentrations listed in the column under the heading "A Preferred Embodiment in Supplement" in Table A below.

Table A: Concentrations of Non-Trace Element Moiety Ingredients

| Ingredient | A preferred embodiment in supplement (mg/L) (About) | Concentration range in 1X medium (mg/L) (About) | A preferred embodiment in 1X medium (mg/L) (About) |
|---|---|---|---|
| Glycine | 150 | 5-200 | 53 |
| L-Histidine | 940 | 5-250 | 183 |
| L-Isoleucine | 3400 | 5-300 | 615 |
| L-Methionine | 90 | 5-200 | 44 |

| | | | |
|---|---|---|---|
| L-Phenylalanine | 1800 | 5-400 | 336 |
| L-Proline | 4000 | 1-1000 | 600 |
| L-Hydroxyproline | 100 | 1-45 | 15 |
| L-Serine | 800 | 1-250 | 162 |
| L-Threonine | 2200 | 10-500 | 425 |
| L-Tryptophan | 440 | 2-110 | 82 |
| L-Tyrosine | 77 | 3-175 | 84 |
| L-Valine | 2400 | 5-500 | 454 |
| Thiamine | 33 | 1-20 | 9 |
| Reduced Glutathione | 10 | 1-20 | 1.5 |
| Ascorbic Acid-2-$PO_4$ (Mg Salt) | 330 | 1-200 | 50 |
| Transferrin (iron saturated) | 55 | 1-50 | 8 |
| Insulin | 100 | 1-100 | 10 |
| Sodium Selenite | 0.07 | 0.000001-0.0001 | 0.00001 |
| AlbuMAX®I | 83,000 | 5000-50,000 | 12,500 |

**[00862]** In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μM), 2-mercaptoethanol (final concentration of about 100 μM).

**[00863]** In some embodiments, the defined media described in Smith, *et al.*, "Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement," *Clin Transl Immunology*, 4(1) 2015 (doi: 10.1038/cti.2014.31) are useful in the present invention. Briefly, RPMI or CTS™ OpTmizer™ was used as the basal cell medium, and supplemented with either 0, 2%, 5%, or 10% CTS™ Immune Cell Serum Replacement.

**[00864]** In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME or βME; also known as 2-mercaptoethanol, CAS 60-24-2).

**[00865]** In an embodiment, the rapid second expansion (including expansions referred to as REP) is performed and further comprises a step wherein TILs are selected for superior tumor

reactivity. Any selection method known in the art may be used. For example, the methods described in U.S. Patent Application Publication No. 2016/0010058 A1, the disclosures of which are incorporated herein by reference, may be used for selection of TILs for superior tumor reactivity.

**[00866]** Optionally, a cell viability assay can be performed after the rapid second expansion (including expansions referred to as the REP expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. In some embodiments, TIL samples can be counted and viability determined using a Cellometer K2 automated cell counter (Nexcelom Bioscience, Lawrence, MA). In some embodiments, viability is determined according to the standard Cellometer K2 Image Cytometer Automatic Cell Counter protocol.

**[00867]** The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained in the second expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCRα/β).

**[00868]** In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below. In

some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises 6000 IU/mL IL-2, 30 ug/flask OKT-3, as well as $7.5 \times 10^8$ antigen-presenting feeder cells (APCs), as discussed in more detail below. In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below. In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises 6000 IU/mL IL-2, 30 ug/flask OKT-3, as well as $5 \times 10^8$ antigen-presenting feeder cells (APCs), as discussed in more detail below.

**[00869]** In some embodiments, the rapid second expansion, for example, Step D according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-100 or a G-REX-500. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-500.

1. Feeder Cells and Antigen Presenting Cells

**[00870]** In an embodiment, the rapid second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as well as those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the rapid second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

**[00871]** In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

**[00872]** In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of

viable cells on day 7 or 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

**[00873]** In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 3000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 60 ng/ml OKT3 antibody and 6000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 60 ng/ml OKT3 antibody and 3000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 6000 IU/ml IL-2.

**[00874]** In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 1000-6000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 2000-5000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 2000-4000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 2500-3500 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 6000 IU/ml IL-2.

**[00875]** In some embodiments, the antigen-presenting feeder cells are PBMCs. In some embodiments, the antigen-presenting feeder cells are artificial antigen-presenting feeder cells. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 10, about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

**[00876]** In an embodiment, the second expansion procedures described herein require a ratio of about $5 \times 10^8$ feeder cells to about $100 \times 10^6$ TILs. In an embodiment, the second expansion procedures described herein require a ratio of about $7.5 \times 10^8$ feeder cells to about $100 \times 10^6$ TILs. In another embodiment, the second expansion procedures described herein require a ratio of about $5 \times 10^8$ feeder cells to about $50 \times 10^6$ TILs. In another embodiment, the second expansion procedures described herein require a ratio of about $7.5 \times 10^8$ feeder cells to about $50 \times 10^6$ TILs. In yet another embodiment, the second expansion procedures described herein require about $5 \times 10^8$ feeder cells to about $25 \times 10^6$ TILs. In yet another embodiment, the second expansion procedures described herein require about $7.5 \times 10^8$ feeder cells to about $25 \times 10^6$ TILs. In yet another embodiment, the rapid second expansion requires twice the number of feeder cells as the rapid second expansion. In yet another embodiment, when the priming first expansion described herein requires about $2.5 \times 10^8$ feeder cells, the rapid second expansion requires about $5 \times 10^8$ feeder cells. In yet another embodiment, when the priming first expansion described herein requires about $2.5 \times 10^8$ feeder cells, the rapid second expansion requires about $7.5 \times 10^8$ feeder cells. In yet another embodiment, the rapid second expansion requires two times (2.0X), 2.5X, 3.0X, 3.5X or 4.0X the number of feeder cells as the priming first expansion.

**[00877]** In an embodiment, the rapid second expansion procedures described herein require an excess of feeder cells during the rapid second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In an embodiment, artificial antigen-presenting (aAPC) cells are used in place of PBMCs. In some embodiments, the PBMCs are added to the rapid second expansion at twice the concentration of PBMCs that were added to the priming first expansion.

**[00878]** In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

**[00879]** In an embodiment, artificial antigen presenting cells are used in the rapid second expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

**[00880]** The rapid second expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

**[00881]** Alternatively, using combinations of cytokines for the rapid second expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in WO 2015/189356 and WO 2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21, and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

### E. STEP E: Harvest TILS

**[00882]** After the rapid second expansion step, cells can be harvested. In some embodiments the TILs are harvested after one, two, three, four or more expansion steps, for example as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments the TILs are harvested after two expansion steps, one priming first expansion and one rapid second expansion, for example as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C).

**[00883]** TILs can be harvested in any appropriate and sterile manner, including, for example by centrifugation. Methods for TIL harvesting are well known in the art and any such known methods can be employed with the present process. In some embodiments, TILS are harvested using an automated system.

**[00884]** Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing system is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term "LOVO cell processing system" also refers to any instrument or device manufactured by any vendor that can pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can

perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

**[00885]** In some embodiments, the rapid second expansion, for example, Step D according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-100 or a G-REX-500. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-500.

**[00886]** In some embodiments, Step E according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed according to the processes described herein. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described herein is employed.

**[00887]** In some embodiments, TILs are harvested according to the methods described in herein. In some embodiments, TILs between days 14 and 16 are harvested using the methods as described herein. In some embodiments, TILs are harvested at 14 days using the methods as described herein. In some embodiments, TILs are harvested at 15 days using the methods as described herein. In some embodiments, TILs are harvested at 16 days using the methods as described herein.

### F. STEP F: Final Formulation/ Transfer to Infusion Bag

**[00888]** After Steps A through E as provided in an exemplary order in Figure 8 (in particular, *e.g.*, Figure 8B) and as outlined in detailed above and herein are complete, cells are transferred to a container for use in administration to a patient. In some embodiments, once a therapeutically sufficient number of TILs are obtained using the expansion methods described above, they are transferred to a container for use in administration to a patient.

**[00889]** In an embodiment, TILs expanded using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In an embodiment, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded as disclosed herein may be administered by any suitable route as known in the art. In some embodiments, the TILs are administered as a single intra-arterial or intravenous infusion,

which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic.

### G. PBMC Feeder Cell Ratios

**[00890]** In some embodiments, the culture media used in expansion methods described herein (see for example, Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) include an anti-CD3 antibody e.g. OKT-3. An anti-CD3 antibody in combination with IL-2 induces T cell activation and cell division in the TIL population. This effect can be seen with full length antibodies as well as Fab and F(ab')2 fragments, with the former being generally preferred; see, *e.g.,* Tsoukas *et al.*, *J. Immunol.* **1985,** *135,* 1719, hereby incorporated by reference in its entirety.

**[00891]** In an embodiment, the number of PBMC feeder layers is calculated as follows:

A. Volume of a T-cell (10 μm diameter): $V = (4/3)\,\pi r^3 = 523.6\ \mu m^3$

B. Columne of G-Rex 100 (M) with a 40 μm (4 cells) height: $V = (4/3)\,\pi r^3 = 4\times10^{12}\ \mu m^3$

C. Number cell required to fill column B: $4\times10^{12}\ \mu m^3 / 523.6\ \mu m^3 = 7.6\times10^{8}\ \mu m^3 * 0.64 = 4.86\times10^{8}$

D. Number cells that can be optimally activated in 4D space: $4.86\times10^{8} / 24 = 20.25\times10^{6}$

E. Number of feeders and TIL extrapolated to G-Rex 500: TIL: $100\times10^{6}$ and Feeder: $2.5\times10^{9}$

In this calculation, an approximation of the number of mononuclear cells required to provide an icosahedral geometry for activation of TIL in a cylinder with a 100 $cm^2$ base is used. The calculation derives the experimental result of ~$5\times10^{8}$ for threshold activation of T-cells which closely mirrors NCI experimental data.[(1)] (C) The multiplier (0.64) is the random packing density for equivalent spheres as calculated by Jaeger and Nagel in 1992 [(2)]. (D) The divisor 24 is the number of equivalent spheres that could contact a similar object in 4 dimensional space "the Newton number."[(3)].

[(1)] Jin, Jianjian, et.al., Simplified Method of the Growth of Human Tumor Infiltrating Lymphocytes (TIL) in Gas-Permeable Flasks to Numbers Needed for Patient Treatment. J Immunother. 2012 Apr; 35(3): 283–292.

[(2)] Jaeger HM, Nagel SR. Physics of the granular state. Science. 1992 Mar 20;255(5051):1523-31.

[3] O. R. Musin (2003). "The problem of the twenty-five spheres". Russ. Math. Surv. 58 (4): 794–795.

**[00892]** In an embodiment, the number of antigen-presenting feeder cells exogenously supplied during the priming first expansion is approximately one-half the number of antigen-presenting feeder cells exogenously supplied during the rapid second expansion. In certain embodiments, the method comprises performing the priming first expansion in a cell culture medium which comprises approximately 50% fewer antigen presenting cells as compared to the cell culture medium of the rapid second expansion.

**[00893]** In another embodiment, the number of antigen-presenting feeder cells (APCs) exogenously supplied during the rapid second expansion is greater than the number of APCs exogenously supplied during the priming first expansion.

**[00894]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 20:1.

**[00895]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 10:1.

**[00896]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 9:1.

**[00897]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 8:1.

**[00898]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 7:1.

**[00899]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 6:1.

**[00900]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 5:1.

**[00901]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 4:1.

**[00902]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion) is selected from a range of from at or about 1.1:1 to at or about 3:1.

**[00903]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.9:1.

**[00904]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.8:1.

**[00905]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.7:1.

**[00906]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.6:1.

**[00907]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.5:1.

**[00908]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.4:1.

**[00909]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.3:1.

**[00910]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.2:1.

**[00911]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.1:1.

**[00912]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2:1.

**[00913]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 10:1.

**[00914]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 5:1.

**[00915]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 4:1.

**[00916]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 3:1.

**[00917]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.9:1.

**[00918]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.8:1.

**[00919]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.7:1.

**[00920]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.6:1.

**[00921]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.5:1.

**[00922]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.4:1.

**[00923]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.3:1.

**[00924]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about about 2:1 to at or about 2.2:1.

**[00925]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.1:1.

**[00926]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is at or about 2:1.

**[00927]** In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is at or about 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, 2:1, 2.1:1, 2.2:1, 2.3:1, 2.4:1, 2.5:1, 2.6:1, 2.7:1, 2.8:1, 2.9:1, 3:1, 3.1:1, 3.2:1, 3.3:1, 3.4:1, 3.5:1, 3.6:1, 3.7:1, 3.8:1, 3.9:1, 4:1, 4.1:1, 4.2:1, 4.3:1, 4.4:1, 4.5:1, 4.6:1, 4.7:1, 4.8:1, 4.9:1, or 5:1.

**[00928]** In another embodiment, the number of APCs exogenously supplied during the priming first expansion is at or about $1\times10^8$, $1.1\times10^8$, $1.2\times10^8$, $1.3\times10^8$, $1.4\times10^8$, $1.5\times10^8$, $1.6\times10^8$, $1.7\times10^8$, $1.8\times10^8$, $1.9\times10^8$, $2\times10^8$, $2.1\times10^8$, $2.2\times10^8$, $2.3\times10^8$, $2.4\times10^8$, $2.5\times10^8$,

$2.6\times10^8$, $2.7\times10^8$, $2.8\times10^8$, $2.9\times10^8$, $3\times10^8$, $3.1\times10^8$, $3.2\times10^8$, $3.3\times10^8$, $3.4\times10^8$ or $3.5\times10^8$ APCs, and the number of APCs exogenously supplied during the rapid second expansion is at or about $3.5\times10^8$, $3.6\times10^8$, $3.7\times10^8$, $3.8\times10^8$, $3.9\times10^8$, $4\times10^8$, $4.1\times10^8$, $4.2\times10^8$, $4.3\times10^8$, $4.4\times10^8$, $4.5\times10^8$, $4.6\times10^8$, $4.7\times10^8$, $4.8\times10^8$, $4.9\times10^8$, $5\times10^8$, $5.1\times10^8$, $5.2\times10^8$, $5.3\times10^8$, $5.4\times10^8$, $5.5\times10^8$, $5.6\times10^8$, $5.7\times10^8$, $5.8\times10^8$, $5.9\times10^8$, $6\times10^8$, $6.1\times10^8$, $6.2\times10^8$, $6.3\times10^8$, $6.4\times10^8$, $6.5\times10^8$, $6.6\times10^8$, $6.7\times10^8$, $6.8\times10^8$, $6.9\times10^8$, $7\times10^8$, $7.1\times10^8$, $7.2\times10^8$, $7.3\times10^8$, $7.4\times10^8$, $7.5\times10^8$, $7.6\times10^8$, $7.7\times10^8$, $7.8\times10^8$, $7.9\times10^8$, $8\times10^8$, $8.1\times10^8$, $8.2\times10^8$, $8.3\times10^8$, $8.4\times10^8$, $8.5\times10^8$, $8.6\times10^8$, $8.7\times10^8$, $8.8\times10^8$, $8.9\times10^8$, $9\times10^8$, $9.1\times10^8$, $9.2\times10^8$, $9.3\times10^8$, $9.4\times10^8$, $9.5\times10^8$, $9.6\times10^8$, $9.7\times10^8$, $9.8\times10^8$, $9.9\times10^8$ or $1\times10^9$ APCs.

**[00929]** In another embodiment, the number of APCs exogenously supplied during the priming first expansion is selected from the range of at or about $1.5\times10^8$ APCs to at or about $3\times10^8$ APCs, and the number of APCs exogenously supplied during the rapid second expansion is selected from the range of at or about $4\times10^8$ APCs to at or about $7.5\times10^8$ APCs.

**[00930]** In another embodiment, the number of APCs exogenously supplied during the priming first expansion is selected from the range of at or about $2\times10^8$ APCs to at or about $2.5\times10^8$ APCs, and the number of APCs exogenously supplied during the rapid second expansion is selected from the range of at or about $4.5\times10^8$ APCs to at or about $5.5\times10^8$ APCs.

**[00931]** In another embodiment, the number of APCs exogenously supplied during the priming first expansion is at or about $2.5\times10^8$ APCs, and the number of APCs exogenously supplied during the rapid second expansion is at or about $5\times10^8$ APCs.

**[00932]** In an embodiment, the number of APCs (including, for example, PBMCs) added at day 0 of the priming first expansion is approximately one-half of the number of PBMCs added at day 7 of the priming first expansion (e.g., day 7 of the method). In certain embodiments, the method comprises adding antigen presenting cells at day 0 of the priming first expansion to the first population of TILs and adding antigen presenting cells at day 7 to the second population of TILs, wherein the number of antigen presenting cells added at day 0 is approximately 50% of the number of antigen presenting cells added at day 7 of the priming first expansion (e.g., day 7 of the method).

**[00933]** In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is greater than the number of PBMCs exogenously supplied at day 0 of the priming first expansion.

**[00934]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about $1.0\times10^6$ APCs/cm$^2$ to at or about $4.5\times10^6$ APCs/cm$^2$.

**[00935]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about $1.5\times10^6$ APCs/cm$^2$ to at or about $3.5\times10^6$ APCs/cm$^2$.

**[00936]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about $2\times10^6$ APCs/cm$^2$ to at or about $3\times10^6$ APCs/cm$^2$.

**[00937]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density of at or about $2\times10^6$ APCs/cm$^2$.

**[00938]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density of at or about $1.0\times10^6$, $1.1\times10^6$, $1.2\times10^6$, $1.3\times10^6$, $1.4\times10^6$, $1.5\times10^6$, $1.6\times10^6$, $1.7\times10^6$, $1.8\times10^6$, $1.9\times10^6$, $2\times10^6$, $2.1\times10^6$, $2.2\times10^6$, $2.3\times10^6$, $2.4\times10^6$, $2.5\times10^6$, $2.6\times10^6$, $2.7\times10^6$, $2.8\times10^6$, $2.9\times10^6$, $3\times10^6$, $3.1\times10^6$, $3.2\times10^6$, $3.3\times10^6$, $3.4\times10^6$, $3.5\times10^6$, $3.6\times10^6$, $3.7\times10^6$, $3.8\times10^6$, $3.9\times10^6$, $4\times10^6$, $4.1\times10^6$, $4.2\times10^6$, $4.3\times10^6$, $4.4\times10^6$ or $4.5\times10^6$ APCs/cm$^2$.

**[00939]** In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $2.5\times10^6$ APCs/cm$^2$ to at or about $7.5\times10^6$ APCs/cm$^2$.

**[00940]** In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $3.5\times10^6$ APCs/cm$^2$ to about $6.0\times10^6$ APCs/cm$^2$.

**[00941]** In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $4.0\times10^6$ APCs/cm$^2$ to about $5.5\times10^6$ APCs/cm$^2$.

**[00942]** In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $4.0\times10^6$ APCs/cm$^2$.

**[00943]** In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density of at or about $2.5\times10^6$ APCs/cm$^2$,

$2.6\times10^6$ APCs/cm$^2$, $2.7\times10^6$ APCs/cm$^2$, $2.8\times10^6$, $2.9\times10^6$, $3\times10^6$, $3.1\times10^6$, $3.2\times10^6$, $3.3\times10^6$, $3.4\times10^6$, $3.5\times10^6$, $3.6\times10^6$, $3.7\times10^6$, $3.8\times10^6$, $3.9\times10^6$, $4\times10^6$, $4.1\times10^6$, $4.2\times10^6$, $4.3\times10^6$, $4.4\times10^6$, $4.5\times10^6$, $4.6\times10^6$, $4.7\times10^6$, $4.8\times10^6$, $4.9\times10^6$, $5\times10^6$, $5.1\times10^6$, $5.2\times10^6$, $5.3\times10^6$, $5.4\times10^6$, $5.5\times10^6$, $5.6\times10^6$, $5.7\times10^6$, $5.8\times10^6$, $5.9\times10^6$, $6\times10^6$, $6.1\times10^6$, $6.2\times10^6$, $6.3\times10^6$, $6.4\times10^6$, $6.5\times10^6$, $6.6\times10^6$, $6.7\times10^6$, $6.8\times10^6$, $6.9\times10^6$, $7\times10^6$, $7.1\times10^6$, $7.2\times10^6$, $7.3\times10^6$, $7.4\times10^6$ or $7.5\times10^6$ APCs/cm$^2$.

**[00944]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density of at or about $1.0\times10^6$, $1.1\times10^6$, $1.2\times10^6$, $1.3\times10^6$, $1.4\times10^6$, $1.5\times10^6$, $1.6\times10^6$, $1.7\times10^6$, $1.8\times10^6$, $1.9\times10^6$, $2\times10^6$, $2.1\times10^6$, $2.2\times10^6$, $2.3\times10^6$, $2.4\times10^6$, $2.5\times10^6$, $2.6\times10^6$, $2.7\times10^6$, $2.8\times10^6$, $2.9\times10^6$, $3\times10^6$, $3.1\times10^6$, $3.2\times10^6$, $3.3\times10^6$, $3.4\times10^6$, $3.5\times10^6$, $3.6\times10^6$, $3.7\times10^6$, $3.8\times10^6$, $3.9\times10^6$, $4\times10^6$, $4.1\times10^6$, $4.2\times10^6$, $4.3\times10^6$, $4.4\times10^6$ or $4.5\times10^6$ APCs/cm$^2$ and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density of at or about $2.5\times10^6$ APCs/cm$^2$, $2.6\times10^6$ APCs/cm$^2$, $2.7\times10^6$ APCs/cm$^2$, $2.8\times10^6$, $2.9\times10^6$, $3\times10^6$, $3.1\times10^6$, $3.2\times10^6$, $3.3\times10^6$, $3.4\times10^6$, $3.5\times10^6$, $3.6\times10^6$, $3.7\times10^6$, $3.8\times10^6$, $3.9\times10^6$, $4\times10^6$, $4.1\times10^6$, $4.2\times10^6$, $4.3\times10^6$, $4.4\times10^6$, $4.5\times10^6$, $4.6\times10^6$, $4.7\times10^6$, $4.8\times10^6$, $4.9\times10^6$, $5\times10^6$, $5.1\times10^6$, $5.2\times10^6$, $5.3\times10^6$, $5.4\times10^6$, $5.5\times10^6$, $5.6\times10^6$, $5.7\times10^6$, $5.8\times10^6$, $5.9\times10^6$, $6\times10^6$, $6.1\times10^6$, $6.2\times10^6$, $6.3\times10^6$, $6.4\times10^6$, $6.5\times10^6$, $6.6\times10^6$, $6.7\times10^6$, $6.8\times10^6$, $6.9\times10^6$, $7\times10^6$, $7.1\times10^6$, $7.2\times10^6$, $7.3\times10^6$, $7.4\times10^6$ or $7.5\times10^6$ APCs/cm$^2$.

**[00945]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about $1.0\times10^6$ APCs/cm$^2$ to at or about $4.5\times10^6$ APCs/cm$^2$, and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $2.5\times10^6$ APCs/cm$^2$ to at or about $7.5\times10^6$ APCs/cm$^2$.

**[00946]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about $1.5\times10^6$ APCs/cm$^2$ to at or about $3.5\times10^6$ APCs/cm$^2$, and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $3.5\times10^6$ APCs/cm$^2$ to at or about $6\times10^6$ APCs/cm$^2$.

**[00947]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about $2\times10^6$ APCs/cm$^2$ to at or about $3\times10^6$ APCs/cm$^2$, and the APCs exogenously supplied in the

rapid second expansion are seeded in the culture flask at a density selected from a range of at or about $4\times10^6$ APCs/$cm^2$ to at or about $5.5\times10^6$ APCs/$cm^2$.

**[00948]** In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density at or about $2\times10^6$ APCs/$cm^2$ and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density of at or about $4\times10^6$ APCs/$cm^2$.

**[00949]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of PBMCs exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 20:1.

**[00950]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of PBMCs exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 10:1.

**[00951]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of PBMCs exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 9:1.

**[00952]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 8:1.

**[00953]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 7:1.

**[00954]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 6:1.

**[00955]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 5:1.

**[00956]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 4:1.

**[00957]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 3:1.

**[00958]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.9:1.

**[00959]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.8:1.

**[00960]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.7:1.

**[00961]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.6:1.

**[00962]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.5:1.

**[00963]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.4:1.

**[00964]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.3:1.

**[00965]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.2:1.

**[00966]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.1:1.

**[00967]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2:1.

**[00968]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 10:1.

**[00969]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 5:1.

**[00970]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 4:1.

**[00971]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 3:1.

**[00972]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.9:1.

**[00973]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.8:1.

**[00974]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.7:1.

**[00975]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.6:1.

**[00976]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.5:1.

**[00977]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.4:1.

**[00978]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.3:1.

**[00979]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about about 2:1 to at or about 2.2:1.

**[00980]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.1:1.

**[00981]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about 2:1.

**[00982]** In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, 2:1, 2.1:1, 2.2:1, 2.3:1, 2.4:1, 2.5:1, 2.6:1, 2.7:1, 2.8:1, 2.9:1, 3:1, 3.1:1, 3.2:1, 3.3:1, 3.4:1, 3.5:1, 3.6:1, 3.7:1, 3.8:1, 3.9:1, 4:1, 4.1:1, 4.2:1, 4.3:1, 4.4:1, 4.5:1, 4.6:1, 4.7:1, 4.8:1, 4.9:1, or 5:1.

**[00983]** In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about $1\times10^8$, $1.1\times10^8$, $1.2\times10^8$, $1.3\times10^8$, $1.4\times10^8$, $1.5\times10^8$, $1.6\times10^8$, $1.7\times10^8$, $1.8\times10^8$, $1.9\times10^8$, $2\times10^8$, $2.1\times10^8$, $2.2\times10^8$, $2.3\times10^8$, $2.4\times10^8$, $2.5\times10^8$, $2.6\times10^8$, $2.7\times10^8$, $2.8\times10^8$, $2.9\times10^8$, $3\times10^8$, $3.1\times10^8$, $3.2\times10^8$, $3.3\times10^8$, $3.4\times10^8$ or $3.5\times10^8$ APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is at or about $3.5\times10^8$, $3.6\times10^8$, $3.7\times10^8$, $3.8\times10^8$, $3.9\times10^8$, $4\times10^8$, $4.1\times10^8$, $4.2\times10^8$, $4.3\times10^8$, $4.4\times10^8$, $4.5\times10^8$, $4.6\times10^8$, $4.7\times10^8$, $4.8\times10^8$, $4.9\times10^8$, $5\times10^8$, $5.1\times10^8$, $5.2\times10^8$, $5.3\times10^8$, $5.4\times10^8$, $5.5\times10^8$, $5.6\times10^8$, $5.7\times10^8$, $5.8\times10^8$, $5.9\times10^8$, $6\times10^8$, $6.1\times10^8$, $6.2\times10^8$, $6.3\times10^8$, $6.4\times10^8$, $6.5\times10^8$, $6.6\times10^8$, $6.7\times10^8$, $6.8\times10^8$, $6.9\times10^8$, $7\times10^8$, $7.1\times10^8$, $7.2\times10^8$, $7.3\times10^8$, $7.4\times10^8$, $7.5\times10^8$, $7.6\times10^8$, $7.7\times10^8$, $7.8\times10^8$, $7.9\times10^8$, $8\times10^8$, $8.1\times10^8$, $8.2\times10^8$, $8.3\times10^8$, $8.4\times10^8$, $8.5\times10^8$, $8.6\times10^8$, $8.7\times10^8$, $8.8\times10^8$, $8.9\times10^8$, $9\times10^8$, $9.1\times10^8$, $9.2\times10^8$, $9.3\times10^8$, $9.4\times10^8$, $9.5\times10^8$, $9.6\times10^8$, $9.7\times10^8$, $9.8\times10^8$, $9.9\times10^8$ or $1x10^9$ APCs (including, for example, PBMCs).

**[00984]** In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from the range of at or about $1\times10^8$ APCs (including, for example, PBMCs) to at or about $3.5\times10^8$ APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is selected from the range of at or about $3.5\times10^8$ APCs (including, for example, PBMCs) to at or about $1\times10^9$ APCs (including, for example, PBMCs).

**[00985]** In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from the range of at or about $1.5\times10^8$ APCs to at or about $3\times10^8$ APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is selected from the range of at or about $4\times10^8$ APCs (including, for example, PBMCs) to at or about $7.5\times10^8$ APCs (including, for example, PBMCs).

**[00986]** In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from the range of at or about $2\times10^8$ APCs (including, for example, PBMCs) to at or about $2.5\times10^8$ APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is selected from the range of at or about $4.5\times10^8$ APCs (including, for example, PBMCs) to at or about $5.5\times10^8$ APCs (including, for example, PBMCs).

**[00987]** In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about $2.5\times10^8$ APCs (including, for example, PBMCs) and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is at or about $5\times10^8$ APCs (including, for example, PBMCs)

**[00988]** In an embodiment, the number of layers of APCs (including, for example, PBMCs) added at day 0 of the priming first expansion is approximately one-half of the number of layers of APCs (including, for example, PBMCs) added at day 7 of the rapid second expansion. In certain embodiments, the method comprises adding antigen presenting cell layers at day 0 of the priming first expansion to the first population of TILs and adding antigen presenting cell layers at day 7 to the second population of TILs, wherein the number

of antigen presenting cell layer added at day 0 is approximately 50% of the number of antigen presenting cell layers added at day 7.

**[00989]** In another embodiment, the number of layers of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is greater than the number of layers of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion.

**[00990]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 4 cell layers.

**[00991]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about one cell layer and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3 cell layers.

**[00992]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 1.5 cell layers to at or about 2.5 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3 cell layers.

**[00993]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about one cell layer and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers.

**[00994]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of of at or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8 cell layers.

**[00995]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 1 cell layer to at or about 2 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3 cell layers to at or about 10 cell layers.

**[00996]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers to at or about 3 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 4 cell layers to at or about 8 cell layers.

**[00997]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 4 cell layers to at or about 8 cell layers.

**[00998]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 1, 2 or 3 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3, 4, 5, 6, 7, 8, 9 or 10 cell layers.

**[00999]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:10.

**[001000]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:8.

**[001001]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:7.

**[001002]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:6.

**[001003]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:5.

**[001004]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:4.

**[001005]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:3.

**[001006]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:2.

**[001007]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.2 to at or about 1:8.

**[001008]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.3 to at or about 1:7.

**[001009]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.4 to at or about 1:6.

**[001010]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.5 to at or about 1:5.

**[001011]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.6 to at or about 1:4.

**[001012]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.7 to at or about 1:3.5.

**[001013]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.8 to at or about 1:3.

**[001014]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.9 to at or about 1:2.5.

**[001015]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is at or about 1: 2.

**[001016]** In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from at or about 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9, 1:2, 1:2.1, 1:2.2, 1:2.3, 1:2.4, 1:2.5, 1:2.6, 1:2.7, 1:2.8, 1:2.9, 1:3, 1:3.1, 1:3.2, 1:3.3, 1:3.4, 1:3.5, 1:3.6, 1:3.7, 1:3.8, 1:3.9, 1:4, 1:4.1, 1:4.2, 1:4.3, 1:4.4, 1:4.5, 1:4.6, 1:4.7, 1:4.8, 1:4.9, 1:5, 1:5.1, 1:5.2, 1:5.3, 1:5.4, 1:5.5, 1:5.6, 1:5.7, 1:5.8, 1:5.9, 1:6, 1:6.1, 1:6.2, 1:6.3, 1:6.4, 1:6.5, 1:6.6, 1:6.7, 1:6.8, 1:6.9, 1:7, 1:7.1, 1:7.2, 1:7.3, 1:7.4, 1:7.5, 1:7.6, 1:7.7, 1:7.8, 1:7.9, 1:8, 1:8.1, 1:8.2, 1:8.3, 1:8.4, 1:8.5, 1:8.6, 1:8.7, 1:8.8, 1:8.9, 1:9, 1:9.1, 1:9.2, 1:9.3, 1:9.4, 1:9.5, 1:9.6, 1:9.7, 1:9.8, 1:9.9 or 1:10.

**[001017]** In some embodiments, the number of APCs in the priming first expansion is selected from the range of about $1.0\times10^6$ APCs/cm$^2$ to about $4.5\times10^6$ APCs/cm$^2$, and the number of APCs in the rapid second expansion is selected from the range of about $2.5\times10^6$ APCs/cm$^2$ to about $7.5\times10^6$ APCs/cm$^2$.

**[001018]** In some embodiments, the number of APCs in the priming first expansion is selected from the range of about $1.5\times10^6$ APCs/cm$^2$ to about $3.5\times10^6$ APCs/cm$^2$, and the number of APCs in the rapid second expansion is selected from the range of about $3.5\times10^6$ APCs/cm$^2$ to about $6.0\times10^6$ APCs/cm$^2$.

**[001019]** In some embodiments, the number of APCs in the priming first expansion is selected from the range of about $2.0\times10^6$ APCs/cm$^2$ to about $3.0\times10^6$ APCs/cm$^2$, and the number of APCs in the rapid second expansion is selected from the range of about $4.0\times10^6$ APCs/cm$^2$ to about $5.5\times10^6$ APCs/cm$^2$.

### H. Optional Cell Medium Components

1. Anti-CD3 Antibodies

**[001020]** In some embodiments, the culture media used in expansion methods described herein (see for example, Figure 8 (in particular, *e.g.*, Figure 8B)) include an anti-CD3 antibody. An anti-CD3 antibody in combination with IL-2 induces T cell activation and cell division in the TIL population. This effect can be seen with full length antibodies as well as

Fab and F(ab')2 fragments, with the former being generally preferred; see, *e.g.,* Tsoukas *et al.*, *J. Immunol.* **1985,** *135,* 1719, hereby incorporated by reference in its entirety.

**[001021]** As will be appreciated by those in the art, there are a number of suitable anti-human CD3 antibodies that find use in the invention, including anti-human CD3 polyclonal and monoclonal antibodies from various mammals, including, but not limited to, murine, human, primate, rat, and canine antibodies. In particular embodiments, the OKT3 anti-CD3 antibody is used (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA).

TABLE 21: Amino acid sequences of muromonab (exemplary OKT-3 antibody)

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:1<br>Muromonab heavy chain | QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY<br>NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA<br>KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL<br>YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN<br>STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE<br>LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:2<br>Muromonab light chain | QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH<br>FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS<br>SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL<br>TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC | 60<br>120<br>180<br>213 |

2. 4-1BB (CD137) AGONISTS

**[001022]** In an embodiment, the cell culture medium of the priming first expansion and/or the rapid second expansion comprises a TNFRSF agonist. In an embodiment, the TNFRSF agonist is a 4-1BB (CD137) agonist. The 4-1BB agonist may be any 4-1BB binding molecule known in the art. The 4-1BB binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian 4-1BB. The 4-1BB agonists or 4-1BB binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The 4-1BB agonist or 4-1BB binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that

bind to 4-1BB. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a humanized antibody. In some embodiments, 4-1BB agonists for use in the presently disclosed methods and compositions include anti-4-1BB antibodies, human anti-4-1BB antibodies, mouse anti-4-1BB antibodies, mammalian anti-4-1BB antibodies, monoclonal anti-4-1BB antibodies, polyclonal anti-4-1BB antibodies, chimeric anti-4-1BB antibodies, anti-4-1BB adnectins, anti-4-1BB domain antibodies, single chain anti-4-1BB fragments, heavy chain anti-4-1BB fragments, light chain anti-4-1BB fragments, anti-4-1BB fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. Agonistic anti-4-1BB antibodies are known to induce strong immune responses. Lee, *et al.*, *PLOS One* **2013**, *8,* e69677. In a preferred embodiment, the 4-1BB agonist is an agonistic, anti-4-1BB humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line). In an embodiment, the 4-1BB agonist is EU-101 (Eutilex Co. Ltd.), utomilumab, or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof. In a preferred embodiment, the 4-1BB agonist is utomilumab or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof.

**[001023]** In a preferred embodiment, the 4-1BB agonist or 4-1BB binding molecule may also be a fusion protein. In a preferred embodiment, a multimeric 4-1BB agonist, such as a trimeric or hexameric 4-1BB agonist (with three or six ligand binding domains), may induce superior receptor (4-1BBL) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

**[001024]** Agonistic 4-1BB antibodies and fusion proteins are known to induce strong immune responses. In a preferred embodiment, the 4-1BB agonist is a monoclonal antibody or fusion protein that binds specifically to 4-1BB antigen in a manner sufficient to reduce toxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion

protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein which abrogates Fc region functionality.

**[001025]** In some embodiments, the 4-1BB agonists are characterized by binding to human 4-1BB (SEQ ID NO:9) with high affinity and agonistic activity. In an embodiment, the 4-1BB agonist is a binding molecule that binds to human 4-1BB (SEQ ID NO:9). In an embodiment, the 4-1BB agonist is a binding molecule that binds to murine 4-1BB (SEQ ID NO:10). The amino acid sequences of 4-1BB antigen to which a 4-1BB agonist or binding molecule binds are summarized in Table 22.

TABLE 22. Amino acid sequences of 4-1BB antigens.

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:9 human 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Homo sapiens) | MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR 60<br>TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC 120<br>CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE 180<br>PGHSPQIISF FLALTSTALL FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG 240<br>CSCRFPEEEE GGCEL 255 |
| SEQ ID NO:10 murine 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Mus musculus) | MGNNCYNVVV IVLLLVGCEK VGAVQNSCDN CQPGTFCRKY NPVCKSCPPS TFSSIGGQPN 60<br>CNICRVCAGY FRFKKFCSST HNAECECIEG FHCLGPQCTR CEKDCRPGQE LTKQGCKTCS 120<br>LGTFNDQNGT GVCRPWTNCS LDGRSVLKTG TTEKDVVCGP PVVSFSPSTT ISVTPEGGPG 180<br>GHSLQVLTLF LALTSALLLA LIFITLLFSV LKWIRKKFPH IFKQPFKKTT GAAQEEDACS 240<br>CRCPQEEEGG GGGYEL 256 |

**[001026]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds human or murine 4-1BB with a $K_D$ of about 100 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 90 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 80 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 70 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 60 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 50 pM or lower, binds human or murine 4-1BB with a $K_D$ of about 40 pM or lower, or binds human or murine 4-1BB with a $K_D$ of about 30 pM or lower.

**[001027]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $8 \times 10^5$ l/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $8.5 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a $k_{assoc}$ of about $9 \times 10^5$ 1/M·s or faster, binds to human or murine 4-1BB with a

$k_{assoc}$ of about $9.5 \times 10^5$ 1/M·s or faster, or binds to human or murine 4-1BB with a $k_{assoc}$ of about $1 \times 10^6$ 1/M·s or faster.

**[001028]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.1 \times 10^{-5}$ 1/s or slower , binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.2 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.3 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.4 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.5 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.6 \times 10^{-5}$ 1/s or slower or binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.7 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.8 \times 10^{-5}$ 1/s or slower, binds to human or murine 4-1BB with a $k_{dissoc}$ of about $2.9 \times 10^{-5}$ 1/s or slower, or binds to human or murine 4-1BB with a $k_{dissoc}$ of about $3 \times 10^{-5}$ 1/s or slower.

**[001029]** In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with an $IC_{50}$ of about 10 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 9 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 8 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 7 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 6 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 5 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 4 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 3 nM or lower, binds to human or murine 4-1BB with an $IC_{50}$ of about 2 nM or lower, or binds to human or murine 4-1BB with an $IC_{50}$ of about 1 nM or lower.

**[001030]** In a preferred embodiment, the 4-1BB agonist is utomilumab, also known as PF-05082566 or MOR-7480, or a fragment, derivative, variant, or biosimilar thereof. Utomilumab is available from Pfizer, Inc. Utomilumab is an immunoglobulin G2-lambda, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor (TNFR) superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of utomilumab are set forth in Table 7. Utomilumab comprises glycosylation sites at Asn59 and Asn292; heavy chain intrachain disulfide bridges at positions 22-96 ($V_H$-$V_L$), 143-199 ($C_H1$-$C_L$), 256-316 ($C_H2$) and 362-420 ($C_H3$); light chain intrachain disulfide bridges at positions 22'-87' ($V_H$-$V_L$) and 136'-195' ($C_H1$-$C_L$); interchain heavy chain-heavy chain disulfide bridges at IgG2A isoform positions 218-218, 219-219, 222-222, and 225-225, at IgG2A/B isoform positions 218-130, 219-219, 222-222, and 225-

225, and at IgG2B isoform positions 219-130 (2), 222-222, and 225-225; and interchain heavy chain-light chain disulfide bridges at IgG2A isoform positions 130-213' (2), IgG2A/B isoform positions 218-213' and 130-213', and at IgG2B isoform positions 218-213' (2). The preparation and properties of utomilumab and its variants and fragments are described in U.S. Patent Nos. 8,821,867; 8,337,850; and 9,468,678, and International Patent Application Publication No. WO 2012/032433 A1, the disclosures of each of which are incorporated by reference herein. Preclinical characteristics of utomilumab are described in Fisher, *et al.*, *Cancer Immunolog. & Immunother.* **2012,** *61,* 1721-33. Current clinical trials of utomilumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02444793, NCT01307267, NCT02315066, and NCT02554812.

**[001031]** In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:11 and a light chain given by SEQ ID NO:12. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively.

**[001032]** In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of utomilumab. In an embodiment, the 4-1BB agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:13, and the 4-1BB agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:14, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$

and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14.

**[001033]** In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:18, SEQ ID NO:19, and SEQ ID NO:20, respectively, and conservative amino acid substitutions thereof.

**[001034]** In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to utomilumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological

product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab.

TABLE 23. Amino acid sequences for 4-1BB agonist antibodies related to utomilumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:11 heavy chain for utomilumab | EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMGK IYPGDSYTNY 60<br>SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCARGY GIFDYWGQGT LVTVSSASTK 120<br>GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVHTFP AVLQSSGLYS 180<br>LSSVVTVPSS NFGTQTYTCN VDHKPSNTKV DKTVERKCCV ECPPCPAPPV AGPSVFLFPP 240<br>KPKDTLMISR TPEVTCVVVD VSHEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTFRVVSV 300<br>LTVVHQDWLN GKEYKCKVSN KGLPAPIEKT ISKTKGQPRE PQVYTLPPSR EEMTKNQVSL 360<br>TCLVKGFYPS DIAVEWESNG QPENNYKTTP PMLDSDGSFF LYSKLTVDKS RWQQGNVFSC 420<br>SVMHEALHNH YTQKSLSLSP G 441 |
| SEQ ID NO:12 light chain for utomilumab | SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER 60<br>FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVLGQ PKAAPSVTLF 120<br>PPSSEELQAN KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTPSKQS NNKYAASSYL 180<br>SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS 214 |
| SEQ ID NO:13 heavy chain variable region for utomilumab | EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMG KIYPGDSYTN 60<br>YSPSFQGQVT ISADKSISTA YLQWSSLKAS DTAMYYCARG YGIFDYWGQ GTLVTVSS 118 |
| SEQ ID NO:14 light chain variable region for utomilumab | SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER 60<br>FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVL 108 |
| SEQ ID NO:15 heavy chain CDR1 for utomilumab | STYWIS 6 |
| SEQ ID NO:16 heavy chain CDR2 for utomilumab | KIYPGDSYTN YSPSFQG 17 |
| SEQ ID NO:17 heavy chain CDR3 for utomilumab | RGYGIFDY 8 |
| SEQ ID NO:18 light chain CDR1 for utomilumab | SGDNIGDQYA H 11 |
| SEQ ID NO:19 light chain CDR2 for utomilumab | QDKNRPS 7 |
| SEQ ID NO:20 light chain CDR3 for utomilumab | ATYTGFGSLA V 11 |

**[001035]** In a preferred embodiment, the 4-1BB agonist is the monoclonal antibody urelumab, also known as BMS-663513 and 20H4.9.h4a, or a fragment, derivative, variant, or biosimilar thereof. Urelumab is available from Bristol-Myers Squibb, Inc., and Creative Biolabs, Inc. Urelumab is an immunoglobulin G4-kappa, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of urelumab are set forth in Table EE. Urelumab comprises N-glycosylation sites at positions 298 (and 298'); heavy chain intrachain disulfide bridges at positions 22-95 ($V_H$-$V_L$), 148-204 ($C_H1$-$C_L$), 262-322

($C_H2$) and 368-426 ($C_H3$) (and at positions 22''-95'', 148''-204'', 262''-322'', and 368''-426''); light chain intrachain disulfide bridges at positions 23'-88' ($V_H$-$V_L$) and 136'-196' ($C_H1$-$C_L$) (and at positions 23'''-88''' and 136'''-196'''); interchain heavy chain-heavy chain disulfide bridges at positions 227-227'' and 230-230''; and interchain heavy chain-light chain disulfide bridges at 135-216' and 135''-216'''. The preparation and properties of urelumab and its variants and fragments are described in U.S. Patent Nos. 7,288,638 and 8,962,804, the disclosures of which are incorporated by reference herein. The preclinical and clinical characteristics of urelumab are described in Segal, *et al.*, *Clin. Cancer Res.* **2016,** *available at* http:/dx.doi.org/ 10.1158/1078-0432.CCR-16-1272. Current clinical trials of urelumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT01775631, NCT02110082, NCT02253992, and NCT01471210.

**[001036]** In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:21 and a light chain given by SEQ ID NO:22. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively.

**[001037]** In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of urelumab. In an embodiment, the 4-1BB agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:23, and the 4-1BB agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:24, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID

NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24.

**[001038]** In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:25, SEQ ID NO:26, and SEQ ID NO:27, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30, respectively, and conservative amino acid substitutions thereof.

**[001039]** In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to urelumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or

different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab.

TABLE 24: Amino acid sequences for 4-1BB agonist antibodies related to urelumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:21 heavy chain for urelumab | QVQLQQWGAG LLKPSETLSL TCAVYGGSFS GYYWSWIRQS PEKGLEWIGE INHGGYVTYN<br>PSLESRVTIS VDTSKNQFSL KLSSVTAADT AVYYCARDYG PGNYDWYFDL WGRGTLVTVS<br>SASTKGPSVF PLAPCSRSTS ESTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS<br>SGLYSLSSVV TVPSSSLGTK TYTCNVDHKP SNTKVDKRVE SKYGPPCPPC PAPEFLGGPS<br>VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST<br>YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT<br>KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE<br>GNVFSCSVMH EALHNHYTQK SLSLSLGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>448 |
| SEQ ID NO:22 light chain for urelumab | EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPALTF CGGTKVEIKR TVAAPSVFIF<br>PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYSLSST<br>LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC | 60<br>120<br>180<br>216 |
| SEQ ID NO:23 variable heavy chain for urelumab | MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YYWSWIRQSP<br>EKGLEWIGEI NHGGYVTYNP SLESRVTISV DTSKNQFSLK LSSVTAADTA VYYCARDYGP | 60<br>120 |
| SEQ ID NO:24 variable light chain for urelumab | MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP<br>GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ | 60<br>110 |
| SEQ ID NO:25 heavy chain CDR1 for urelumab | GYYWS | 5 |
| SEQ ID NO:26 heavy chain CDR2 for urelumab | EINHGGYVTY NPSLES | 16 |
| SEQ ID NO:27 heavy chain CDR3 for urelumab | DYGPGNYDWY FDL | 13 |
| SEQ ID NO:28 light chain CDR1 for urelumab | RASQSVSSYL A | 11 |
| SEQ ID NO:29 light chain CDR2 for urelumab | DASNRAT | 7 |
| SEQ ID NO:30 light chain CDR3 for urelumab | QQRSDWPPAL T | 11 |

**[001040]** In an embodiment, the 4-1BB agonist is selected from the group consisting of 1D8, 3Elor, 4B4 (BioLegend 309809), H4-1BB-M127 (BD Pharmingen 552532), BBK2 (Thermo Fisher MS621PABX), 145501 (Leinco Technologies B591), the antibody produced by cell line deposited as ATCC No. HB-11248 and disclosed in U.S. Patent No. 6,974,863, 5F4 (BioLegend 31 1503), C65-485 (BD Pharmingen 559446), antibodies disclosed in U.S. Patent Application Publication No. US 2005/0095244, antibodies disclosed in U.S. Patent No. 7,288,638 (such as 20H4.9-IgGl (BMS-663031)), antibodies disclosed in U.S. Patent No. 6,887,673 (such as 4E9 or BMS-554271), antibodies disclosed in U.S. Patent No. 7,214,493,

antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in U.S. Patent No. 6,905,685 (such as 4E9 or BMS-554271), antibodies disclosed in U.S. Patent No. 6,362,325 (such as 1D8 or BMS-469492; 3H3 or BMS-469497; or 3El), antibodies disclosed in U.S. Patent No. 6,974,863 (such as 53A2); antibodies disclosed in U.S. Patent No. 6,210,669 (such as 1D8, 3B8, or 3El), antibodies described in U.S. Patent No. 5,928,893, antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in International Patent Application Publication Nos. WO 2012/177788, WO 2015/119923, and WO 2010/042433, and fragments, derivatives, conjugates, variants, or biosimilars thereof, wherein the disclosure of each of the foregoing patents or patent application publications is incorporated by reference here.

**[001041]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein described in International Patent Application Publication Nos. WO 2008/025516 A1, WO 2009/007120 A1, WO 2010/003766 A1, WO 2010/010051 A1, and WO 2010/078966 A1; U.S. Patent Application Publication Nos. US 2011/0027218 A1, US 2015/0126709 A1, US 2011/0111494 A1, US 2015/0110734 A1, and US 2015/0126710 A1; and U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

**[001042]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof (See, Figure 50). In structures I-A and I-B, the cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from *e.g.*, 4-1BBL (4-1BB ligand, CD137 ligand (CD137L), or tumor necrosis factor superfamily member 9 (TNFSF9)) or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second triavelent protein through IgG1-Fc (including $C_H3$ and $C_H2$ domains) is then used to link two of the trivalent proteins together through disulfide bonds (small elongated ovals), stabilizing the structure and providing an agonists capable of bringing together the intracellular signaling domains of the six receptors and signaling proteins to form a signaling complex. The TNFRSF binding domains denoted as cylinders may be scFv domains comprising, *e.g.*, a $V_H$ and a $V_L$ chain connected by a linker that may comprise hydrophilic residues and Gly and Ser sequences for flexibility, as well as Glu and Lys for solubility. Any scFv domain design may

be used, such as those described in de Marco, *Microbial Cell Factories*, **2011,** *10*, 44; Ahmad, *et al.*, *Clin. & Dev. Immunol.* **2012,** 980250; Monnier, *et al.*, *Antibodies,* **2013,** *2,* 193-208; or in references incorporated elsewhere herein. Fusion protein structures of this form are described in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

**[001043]** Amino acid sequences for the other polypeptide domains of structure I-A are given in Table 9. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides.

TABLE 25: Amino acid sequences for TNFRSF agonist fusion proteins, including 4-1BB agonist fusion proteins, with C-terminal Fc-antibody fragment fusion protein design (structure I-A).

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:31<br>Fc domain | KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW<br>YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS<br>KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV<br>LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>230 |
| SEQ ID NO:32<br>linker | GGPGSSKSCD KTHTCPPCPA PE | 22 |
| SEQ ID NO:33<br>linker | GGSGSSKSCD KTHTCPPCPA PE | 22 |
| SEQ ID NO:34<br>linker | GGPGSSSSSS SKSCDKTHTC PPCPAPE | 27 |
| SEQ ID NO:35<br>linker | GGSGSSSSSS SKSCDKTHTC PPCPAPE | 27 |
| SEQ ID NO:36<br>linker | GGPGSSSSSS SSSKSCDKTH TCPPCPAPE | 29 |
| SEQ ID NO:37<br>linker | GGSGSSSSSS SSSKSCDKTH TCPPCPAPE | 29 |
| SEQ ID NO:38<br>linker | GGPGSSGSGS SDKTHTCPPC PAPE | 24 |
| SEQ ID NO:39<br>linker | GGPGSSGSGS DKTHTCPPCP APE | 23 |
| SEQ ID NO:40<br>linker | GGPSSSGSDK THTCPPCPAP E | 21 |
| SEQ ID NO:41<br>linker | GGSSSSSSSS GSDKTHTCPP CPAPE | 25 |

**[001044]** Amino acid sequences for the other polypeptide domains of structure I-B are given in Table 10. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

TABLE 26: Amino acid sequences for TNFRSF agonist fusion proteins, including 4-1BB agonist fusion proteins, with N-terminal Fc-antibody fragment fusion protein design (structure I-B).

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:42<br>Fc domain | METDTLLLWV LLLWVPAGNG DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT<br>CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK<br>CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE<br>WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS<br>LSLSPG | 60<br>120<br>180<br>240<br>246 |
| SEQ ID NO:43<br>linker | SGSGSGSGSG S | 11 |
| SEQ ID NO:44<br>linker | SSSSSSGSGS GS | 12 |
| SEQ ID NO:45<br>linker | SSSSSSGSGS GSGSGS | 16 |

**[001045]** In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains selected from the group consisting of a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain of urelumab, a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table 10, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

**[001046]** In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:46. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a soluble 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:47.

**[001047]** In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an

embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the $V_H$ and $V_L$ sequences given in Table 11, wherein the $V_H$ and $V_L$ domains are connected by a linker.

TABLE 27: Additional polypeptide domains useful as 4-1BB binding domains in fusion proteins or as scFv 4-1BB agonist antibodies.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:46 4-1BBL | MEYASDASLD PEAPWPPAPR ARACRVLPWA LVAGLLLLLL LAAACAVFLA CPWAVSGARA<br>SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL<br>TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAALA<br>LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ GATVLGLFRV<br>TPEIPAGLPS PRSE | 60<br>120<br>180<br>240<br>254 |
| SEQ ID NO:47 4-1BBL soluble domain | LRQGMFAQLV AQNVLLIDGP LSWYSDPGLA GVSLTGGLSY KEDTKELVVA KAGVYYVFFQ<br>LELRRVVAGE GSGSVSLALH LQPLRSAAGA AALALTVDLP PASSEARNSA FGFQGRLLHL<br>SAGQRLGVHL HTEARARHAW QLTQGATVLG LFRVTPEIPA GLPSPRSE | 60<br>120<br>168 |
| SEQ ID NO:48 variable heavy chain for 4B4-1-1 version 1 | QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY<br>NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVS | 60<br>118 |
| SEQ ID NO:49 variable light chain for 4B4-1-1 version 1 | DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS<br>RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIK | 60<br>107 |
| SEQ ID NO:50 variable heavy chain for 4B4-1-1 version 2 | QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY<br>NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVSA | 60<br>119 |
| SEQ ID NO:51 variable light chain for 4B4-1-1 version 2 | DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS<br>RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIKR | 60<br>108 |
| SEQ ID NO:52 variable heavy chain for H39E3-2 | MDWTWRILFL VAAATGAHSE VQLVESGGGL VQPGGSLRLS CAASGFTFSD YWMSWVRQAP<br>GKGLEWVADI KNDGSYTNYA PSLTNRFTIS RDNAKNSLYL QMNSLRAEDT AVYYCARELT | 60<br>120 |
| SEQ ID NO:53 variable light chain for H39E3-2 | MEAPAQLLFL LLLWLPDTTG DIVMTQSPDS LAVSLGERAT INCKSSQSLL SSGNQKNYL<br>WYQQKPGQPP KLLIYYASTR QSGVPDRFSG SGSGTDFTLT ISSLQAEDVA | 60<br>110 |

**[001048]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain, wherein each of the soluble 4-1BB domains lacks a stalk region (which contributes to trimerisation and provides a

certain distance to the cell membrane, but is not part of the 4-1BB binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

**[001049]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain, (iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein each TNF superfamily cytokine domain is a 4-1BB binding domain.

**[001050]** In an embodiment, the 4-1BB agonist is a 4-1BB agonistic scFv antibody comprising any of the foregoing $V_H$ domains linked to any of the foregoing $V_L$ domains.

**[001051]** In an embodiment, the 4-1BB agonist is BPS Bioscience 4-1BB agonist antibody catalog no. 79097-2, commercially available from BPS Bioscience, San Diego, CA, USA. In an embodiment, the 4-1BB agonist is Creative Biolabs 4-1BB agonist antibody catalog no. MOM-18179, commercially available from Creative Biolabs, Shirley, NY, USA.

3. OX40 (CD134) AGONISTS

**[001052]** In an embodiment, the TNFRSF agonist is an OX40 (CD134) agonist. The OX40 agonist may be any OX40 binding molecule known in the art. The OX40 binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian OX40. The OX40 agonists or OX40 binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The OX40 agonist or OX40 binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to OX40. In an embodiment, the OX40 agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the OX40 agonist is an antigen binding protein that is a humanized antibody. In some embodiments, OX40 agonists for use in the presently disclosed methods and compositions include anti-

OX40 antibodies, human anti-OX40 antibodies, mouse anti-OX40 antibodies, mammalian anti-OX40 antibodies, monoclonal anti-OX40 antibodies, polyclonal anti-OX40 antibodies, chimeric anti-OX40 antibodies, anti-OX40 adnectins, anti-OX40 domain antibodies, single chain anti-OX40 fragments, heavy chain anti-OX40 fragments, light chain anti-OX40 fragments, anti-OX40 fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. In a preferred embodiment, the OX40 agonist is an agonistic, anti-OX40 humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line).

**[001053]** In a preferred embodiment, the OX40 agonist or OX40 binding molecule may also be a fusion protein. OX40 fusion proteins comprising an Fc domain fused to OX40L are described, for example, in Sadun, *et al.*, *J. Immunother.* **2009,** *182,* 1481-89. In a preferred embodiment, a multimeric OX40 agonist, such as a trimeric or hexameric OX40 agonist (with three or six ligand binding domains), may induce superior receptor (OX40L) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

**[001054]** Agonistic OX40 antibodies and fusion proteins are known to induce strong immune responses. Curti, *et al.*, *Cancer Res.* **2013**, *73,* 7189-98. In a preferred embodiment, the OX40 agonist is a monoclonal antibody or fusion protein that binds specifically to OX40 antigen in a manner sufficient to reduce toxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein which abrogates Fc region functionality.

**[001055]** In some embodiments, the OX40 agonists are characterized by binding to human OX40 (SEQ ID NO:54) with high affinity and agonistic activity. In an embodiment, the OX40 agonist is a binding molecule that binds to human OX40 (SEQ ID NO:54). In an embodiment, the OX40 agonist is a binding molecule that binds to murine OX40 (SEQ ID

NO:55). The amino acid sequences of OX40 antigen to which an OX40 agonist or binding molecule binds are summarized in Table 12.

TABLE 28: Amino acid sequences of OX40 antigens.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:54<br>human OX40<br>(Homo sapiens) | MCVGARRLGR GPCAALLLLG LGLSTVTGLH CVGDTYPSND RCCHECRPGN GMVSRCSRSQ<br>NTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSERKQLCT ATQDTVCRCR AGTQPLDSYK<br>PGVDCAPCPP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQ<br>GPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL<br>RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI | 60<br>120<br>180<br>240<br>277 |
| SEQ ID NO:55<br>murine OX40<br>(Mus musculus) | MYVWVQQPTA LLLLGLTLGV TARRLNCVKH TYPSGHKCCR ECQPGHGMVS RCDHTRDTLC<br>HPCETGFYNE AVNYDTCKQC TQCNHRSGSE LKQNCTPTQD TVCRCRPGTQ PRQDSGYKLG<br>VDCVPCPPGH FSPGNNQACK PWTNCTLSGK QTRHPASDSL DAVCEDRSLL ATLLWETQRP<br>TFRPTTVQST TVWPRTSELP SPPTLVTPEG PAFAVLLGLG LGLLAPLTVL LALYLLRKAW<br>RLPNTPKPCW GNSFRTPIQE EHTDAHFTLA KI | 60<br>120<br>180<br>240<br>272 |

**[001056]** In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds human or murine OX40 with a $K_D$ of about 100 pM or lower, binds human or murine OX40 with a $K_D$ of about 90 pM or lower, binds human or murine OX40 with a $K_D$ of about 80 pM or lower, binds human or murine OX40 with a $K_D$ of about 70 pM or lower, binds human or murine OX40 with a $K_D$ of about 60 pM or lower, binds human or murine OX40 with a $K_D$ of about 50 pM or lower, binds human or murine OX40 with a $K_D$ of about 40 pM or lower, or binds human or murine OX40 with a $K_D$ of about 30 pM or lower.

**[001057]** In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $8 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $8.5 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $9 \times 10^5$ 1/M·s or faster, binds to human or murine OX40 with a $k_{assoc}$ of about $9.5 \times 10^5$ 1/M·s or faster, or binds to human or murine OX40 with a $k_{assoc}$ of about $1 \times 10^6$ 1/M·s or faster.

**[001058]** In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a $k_{dissoc}$ of about $2 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.1 \times 10^{-5}$ 1/s or slower , binds to human or murine OX40 with a $k_{dissoc}$ of about $2.2 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.3 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.4 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.5 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.6 \times 10^{-5}$ 1/s or slower or binds to human or murine OX40 with a $k_{dissoc}$ of about $2.7 \times 10^{-5}$ 1/s or slower, binds to human or murine OX40 with a $k_{dissoc}$ of about $2.8 \times 10^{-5}$ 1/s or slower, binds

to human or murine OX40 with a $k_{dissoc}$ of about $2.9 \times 10^{-5}$ 1/s or slower, or binds to human or murine OX40 with a $k_{dissoc}$ of about $3 \times 10^{-5}$ 1/s or slower.

**[001059]** In some embodiments, the compositions, processes and methods described include OX40 agonist that binds to human or murine OX40 with an $IC_{50}$ of about 10 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 9 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 8 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 7 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 6 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 5 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 4 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 3 nM or lower, binds to human or murine OX40 with an $IC_{50}$ of about 2 nM or lower, or binds to human or murine OX40 with an $IC_{50}$ of about 1 nM or lower.

**[001060]** In some embodiments, the OX40 agonist is tavolixizumab, also known as MEDI0562 or MEDI-0562. Tavolixizumab is available from the MedImmune subsidiary of AstraZeneca, Inc. Tavolixizumab is immunoglobulin G1-kappa, anti-[*Homo sapiens* TNFRSF4 (tumor necrosis factor receptor (TNFR) superfamily member 4, OX40, CD134)], humanized and chimeric monoclonal antibody. The amino acid sequences of tavolixizumab are set forth in Table 13. Tavolixizumab comprises N-glycosylation sites at positions 301 and 301'', with fucosylated complex bi-antennary CHO-type glycans; heavy chain intrachain disulfide bridges at positions 22-95 ($V_H$-$V_L$), 148-204 ($C_H1$-$C_L$), 265-325 ($C_H2$) and 371-429 ($C_H3$) (and at positions 22''-95'', 148''-204'', 265''-325'', and 371''-429''); light chain intrachain disulfide bridges at positions 23'-88' ($V_H$-$V_L$) and 134'-194' ($C_H1$-$C_L$) (and at positions 23'''-88''' and 134'''-194'''); interchain heavy chain-heavy chain disulfide bridges at positions 230-230'' and 233-233''; and interchain heavy chain-light chain disulfide bridges at 224-214' and 224''-214'''. Current clinical trials of tavolixizumab in a variety of solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02318394 and NCT02705482.

**[001061]** In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:56 and a light chain given by SEQ ID NO:57. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist

comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively.

**[001062]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of tavolixizumab. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:58, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:59, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, an OX40 agonist comprises an scFv antibody comprising $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59.

**[001063]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:63, SEQ ID NO:64, and SEQ ID NO:65, respectively, and conservative amino acid substitutions thereof.

**[001064]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to tavolixizumab. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising

an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab.

TABLE 29: Amino acid sequences for OX40 agonist antibodies related to tavolixizumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:56 heavy chain for tavolixizumab | QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN 60<br>PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVTVS 120<br>SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS 180<br>SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPELLG 240<br>GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY 300<br>NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE 360<br>EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR 420<br>WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 451 |
| SEQ ID NO:57 light chain for tavolixizumab | DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS 60<br>RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKRTV AAPSVFIFPP 120<br>SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT 180<br>LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC 214 |
| SEQ ID NO:58 heavy chain variable region for tavolixizumab | QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN 60<br>PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVT 118 |
| SEQ ID NO:59 light chain variable region for tavolixizumab | DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS 60<br>RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKR 108 |

| | | |
|---|---|---|
| SEQ ID NO:60 heavy chain CDR1 for tavolixizumab | GSFSSGYWN | 9 |
| SEQ ID NO:61 heavy chain CDR2 for tavolixizumab | YIGYISYNGI TYH | 13 |
| SEQ ID NO:62 heavy chain CDR3 for tavolixizumab | RYKYDYDGGH AMDY | 14 |
| SEQ ID NO:63 light chain CDR1 for tavolixizumab | QDISNYLN | 8 |
| SEQ ID NO:64 light chain CDR2 for tavolixizumab | LLIYYTSKLH S | 11 |
| SEQ ID NO:65 light chain CDR3 for tavolixizumab | QQGSALPW | 8 |

**[001065]** In some embodiments, the OX40 agonist is 11D4, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 11D4 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 11D4 are set forth in Table 14.

**[001066]** In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:66 and a light chain given by SEQ ID NO:67. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively.

**[001067]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 11D4. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:68, and the OX40 agonist light

chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:69, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively.

**[001068]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:72, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:73, SEQ ID NO:74, and SEQ ID NO:75, respectively, and conservative amino acid substitutions thereof.

**[001069]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 11D4. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or

more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4.

TABLE 30: Amino acid sequences for OX40 agonist antibodies related to 11D4.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:66 heavy chain for 11D4 | EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSSAS<br>TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL<br>YSLSSVVTVP SSNFGTQTYT CNVDHKPSNT KVDKTVERKC CVECPPCPAP PVAGPSVFLF<br>PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTFRVV<br>SVLTVVHQDW LNGKEYKCKV SNKGLPAPIE KTISKTKGQP REPQVYTLPP SREEMTKNQV<br>SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPMLDSDGS FFLYSKLTVD KSRWQQGNVF<br>SCSVMHEALH NHYTQKSLSL SPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>444 |
| SEQ ID NO:67 light chain for 11D4 | DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS<br>RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIKRTV AAPSVFIFPP<br>SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT<br><br>LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC | 60<br>120<br>180<br><br>214 |
| SEQ ID NO:68 heavy chain variable region for 11D4 | EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSS | 60<br>118 |
| SEQ ID NO:69 light chain variable region for 11D4 | DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS<br>RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIK | 60<br>107 |
| SEQ ID NO:70 heavy chain CDR1 for 11D4 | SYSMN | 5 |
| SEQ ID NO:71 heavy chain CDR2 for 11D4 | YISSSSSTID YADSVKG | 17 |
| SEQ ID NO:72 heavy chain CDR3 for 11D4 | ESGWYLFDY | 9 |
| SEQ ID NO:73 light chain CDR1 for 11D4 | RASQGISSWL A | 11 |
| SEQ ID NO:74 light chain CDR2 for 11D4 | AASSLQS | 7 |
| SEQ ID NO:75 light chain CDR3 for 11D4 | QQYNSYPPT | 9 |

**[001070]** In some embodiments, the OX40 agonist is 18D8, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 18D8 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 18D8 are set forth in Table 15.

**[001071]** In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:76 and a light chain given by SEQ ID NO:77. In an embodiment, a OX40 agonist

comprises heavy and light chains having the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively.

**[001072]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 18D8. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:78, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:79, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively.

**[001073]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:80, SEQ ID NO:81, and SEQ ID NO:82, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:83, SEQ ID NO:84, and SEQ ID NO:85, respectively, and conservative amino acid substitutions thereof.

**[001074]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 18D8. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8.

TABLE 31: Amino acid sequences for OX40 agonist antibodies related to 18D8.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:76 heavy chain for 18D8 | EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV<br>TVSSASTKGP SVFPLAPCSR STSESTAALG CLVKDYFPEP VTVSWNSGAL TSGVHTFPAV<br>LQSSGLYSLS SVVTVPSSNF GTQTYTCNVD HKPSNTKVDK TVERKCCVEC PPCPAPPVAG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVQFNW YVDGVEVHNA KTKPREEQFN<br>STFRVVSVLT VVHQDWLNGK EYKCKVSNKG LPAPIEKTIS KTKGQPREPQ VYTLPPSREE<br>MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPM LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:77 light chain for 18D8 | EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIKRTVA APSVFIFPPS<br>DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL<br>SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC | 60<br>120<br>180<br>213 |
| SEQ ID NO:78 heavy chain variable region for 18D8 | EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY<br>ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV<br>TVSS | 60<br>120<br>124 |
| SEQ ID NO:79 light chain | EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIK | 60<br>106 |

| | | |
|---|---|---|
| variable region for 18D8 | | |
| SEQ ID NO:80 heavy chain CDR1 for 18D8 | DYAMH | 5 |
| SEQ ID NO:81 heavy chain CDR2 for 18D8 | GISWNSGSIG YADSVKG | 17 |
| SEQ ID NO:82 heavy chain CDR3 for 18D8 | DQSTADYYFY YGMDV | 15 |
| SEQ ID NO:83 light chain CDR1 for 18D8 | RASQSVSSYL A | 11 |
| SEQ ID NO:84 light chain CDR2 for 18D8 | DASNRAT | 7 |
| SEQ ID NO:85 light chain CDR3 for 18D8 | QQRSNWPT | 8 |

**[001075]** In some embodiments, the OX40 agonist is Hu119-122, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu119-122 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu119-122 are set forth in Table 16.

**[001076]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu119-122. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:86, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:87, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively.

**[001077]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:90, respectively, and conservative amino acid substitutions thereof, and light chain

CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:91, SEQ ID NO:92, and SEQ ID NO:93, respectively, and conservative amino acid substitutions thereof.

**[001078]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu119-122. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122.

TABLE 32: Amino acid sequences for OX40 agonist antibodies related to Hu119-122.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:86 heavy chain variable region for Hu119-122 | EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY<br>PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS | 60<br>120 |
| SEQ ID NO:87 light chain variable region for Hu119-122 | EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES<br>GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K | 60<br>111 |
| SEQ ID NO:88 heavy chain CDRl for Hu119-122 | SHDMS | 5 |

| | | |
|---|---|---|
| SEQ ID NO:89 heavy chain CDR2 for Hu119-122 | AINSDGGSTY YPDTMER | 17 |
| SEQ ID NO:90 heavy chain CDR3 for Hu119-122 | HYDDYYAWFA Y | 11 |
| SEQ ID NO:91 light chain CDR1 for Hu119-122 | RASKSVSTSG YSYMH | 15 |
| SEQ ID NO:92 light chain CDR2 for Hu119-122 | LASNLES | 7 |
| SEQ ID NO:93 light chain CDR3 for Hu119-122 | QHSRELPLT | 9 |

**[001079]** In some embodiments, the OX40 agonist is Hu106-222, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu106-222 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu106-222 are set forth in Table 17.

**[001080]** In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu106-222. In an embodiment, the OX40 agonist heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:94, and the OX40 agonist light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:95, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively.

**[001081]** In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:99, SEQ ID NO:100, and SEQ ID NO:101, respectively, and conservative amino acid substitutions thereof.

**[001082]** In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu106-222. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222.

TABLE 33: Amino acid sequences for OX40 agonist antibodies related to Hu106-222.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:94 heavy chain variable region for Hu106-222 | QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY<br>ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV<br>SS | 60<br>120<br>122 |
| SEQ ID NO:95 light chain variable region for Hu106-222 | DIQMTQSPSS LSASVGDRVT ITCKASQDVS TAVAWYQQKP GKAPKLLIYS ASYLYTGVPS<br>RFSGSGSGTD FTFTISSLQP EDIATYYCQQ HYSTPRTFGQ GTKLEIK | 60<br>107 |
| SEQ ID NO:96 heavy chain CDR1 for Hu106-222 | DYSMH | 5 |
| SEQ ID NO:97 heavy chain CDR2 for Hu106-222 | WINTETGEPT YADDFKG | 17 |

| | | |
|---|---|---|
| SEQ ID NO:98 heavy chain CDR3 for Hu106-222 | PYYDYVSYYA MDY | 13 |
| SEQ ID NO:99 light chain CDR1 for Hu106-222 | KASQDVSTAV A | 11 |
| SEQ ID NO:100 light chain CDR2 for Hu106-222 | SASYLYT | 7 |
| SEQ ID NO:101 light chain CDR3 for Hu106-222 | QQHYSTPRT | 9 |

**[001083]** In some embodiments, the OX40 agonist antibody is MEDI6469 (also referred to as 9B12). MEDI6469 is a murine monoclonal antibody. Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585. In some embodiments the OX40 agonist is an antibody produced by the 9B12 hybridoma, deposited with Biovest Inc. (Malvern, MA, USA), as described in Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585, the disclosure of which is hereby incorporated by reference in its entirety. In some embodiments, the antibody comprises the CDR sequences of MEDI6469. In some embodiments, the antibody comprises a heavy chain variable region sequence and/or a light chain variable region sequence of MEDI6469.

**[001084]** In an embodiment, the OX40 agonist is L106 BD (Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises the CDRs of antibody L106 (BD Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody L106 (BD Pharmingen Product #340420). In an embodiment, the OX40 agonist is ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises the CDRs of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In an embodiment, the OX40 agonist is the murine monoclonal antibody anti-mCD134/mOX40 (clone OX86), commercially available from InVivoMAb, BioXcell Inc, West Lebanon, NH.

**[001085]** In an embodiment, the OX40 agonist is selected from the OX40 agonists described in International Patent Application Publication Nos. WO 95/12673, WO 95/21925, WO 2006/121810, WO 2012/027328, WO 2013/028231, WO 2013/038191, and WO 2014/148895; European Patent Application EP 0672141; U.S. Patent Application Publication Nos. US 2010/136030, US 2014/377284, US 2015/190506, and US 2015/132288 (including clones 20E5 and 12H3); and U.S. Patent Nos. 7,504,101, 7,550,140, 7,622,444, 7,696,175,

7,960,515, 7,961,515, 8,133,983, 9,006,399, and 9,163,085, the disclosure of each of which is incorporated herein by reference in its entirety.

**[001086]** In an embodiment, the OX40 agonist is an OX40 agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof. The properties of structures I-A and I-B are described above and in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein. Amino acid sequences for the polypeptide domains of structure I-A are given in Table 9. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides. Likewise, amino acid sequences for the polypeptide domains of structure I-B are given in Table 10. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

**[001087]** In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains selected from the group consisting of a variable heavy chain and variable light chain of tavolixizumab, a variable heavy chain and variable light chain of 11D4, a variable heavy chain and variable light chain of 18D8, a variable heavy chain and variable light chain of Hu119-122, a variable heavy chain and variable light chain of Hu106-222, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table 17, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

**[001088]** In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising an OX40L sequence. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:102. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a soluble OX40L sequence. In an

embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:103. In an embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:104.

**[001089]** In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively, wherein the $V_H$ and $V_L$ domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising $V_H$ and $V_L$ regions that are each at least 95% identical to the $V_H$ and $V_L$ sequences given in Table 14, wherein the $V_H$ and $V_L$ domains are connected by a linker.

TABLE 34: Additional polypeptide domains useful as OX40 binding domains in fusion proteins (*e.g.*, structures I-A and I-B) or as scFv OX40 agonist antibodies.

| Identifier | Sequence (One-Letter Amino Acid Symbols) |
|---|---|
| SEQ ID NO:102<br>OX40L | MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL QVSHRYPRIQ 60<br>SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYFS QEVNISLHYQ 120<br>KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF 180<br>CVL 183 |

| | | |
|---|---|---|
| SEQ ID NO:103 OX40L soluble domain | SHRYPRIQSI KVQFTEYKKE KGFILTSQKE DEIMKVQNNS VIINCDGFYL ISLKGYFSQE<br>VNISLHYQKD EEPLFQLKKV RSVNSLMVAS LTYKDKVYLN VTTDNTSLDD FHVNGGELIL<br>IHQNPGEFCV L | 60<br>120<br>131 |
| SEQ ID NO:104 OX40L soluble domain (alternative) | YPRIQSIKVQ FTEYKKEKGF ILTSQKEDEI MKVQNNSVII NCDGFYLISL KGYFSQEVNI<br>SLHYQKDEEP LFQLKKVRSV NSLMVASLTY KDKVYLNVTT DNTSLDDFHV NGGELILIHQ<br>NPGEFCVL | 60<br>120<br>128 |
| SEQ ID NO:105 variable heavy chain for 008 | EVQLVESGGG LVQPGGSLRL SCAASGFTFS NYTMNWVRQA PGKGLEWVSA ISGSGGSTYY<br>ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YSQVHYALDY WGQGTLVTVS | 60<br>120 |
| SEQ ID NO:106 variable light chain for 008 | DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA<br>SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK | 60<br>108 |
| SEQ ID NO:107 variable heavy chain for 011 | EVQLVESGGG VVQPGRSLRL SCAASGFTFS DYTMNWVRQA PGKGLEWVSS ISGGSTYYAD<br>SRKGRFTISR DNSKNTLYLQ MNNLRAEDTA VYYCARDRYF RQQNAFDYWG QGTLVTVSSA | 60<br>120 |
| SEQ ID NO:108 variable light chain for 011 | DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA<br>SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK | 60<br>108 |
| SEQ ID NO:109 variable heavy chain for 021 | EVQLVESGGG LVQPRGSLRL SCAASGFTFS SYAMNWVRQA PGKGLEWVAV ISYDGSNKYY<br>ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YITLPNALDY WGQGTLVTVS | 60<br>120 |
| SEQ ID NO:110 variable light chain for 021 | DIQMTQSPVS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKPGQSPQ LLIYLGSNRA<br>SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYKSNP PTFGQGTK | 60<br>108 |
| SEQ ID NO:111 variable heavy chain for 023 | EVQLVESGGG LVHPGGSLRL SCAGSGFTFS SYAMHWVRQA PGKGLEWVSA IGTGGGTYYA<br>DSVMGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCARYDN VMGLYWFDYW GQGTLVTVSS | 60<br>120 |
| SEQ ID NO:112 variable light chain for 023 | EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPAFGG GTKVEIKR | 60<br>108 |
| SEQ ID NO:113 heavy chain variable region | EVQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY INPYNDGTKY<br>NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCANYY GSSLSMDYWG QGTSVTVSS | 60<br>119 |
| SEQ ID NO:114 light chain variable region | DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS<br>RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTLPWTFGG GTKLEIKR | 60<br>108 |
| SEQ ID NO:115 heavy chain variable region | EVQLQQSGPE LVKPGASVKI SCKTSGYTFK DYTMHWVKQS HGKSLEWIGG IYPNNGGSTY<br>NQNFKDKATL TVDKSSSTAY MEFRSLTSED SAVYYCARMG YHGPHLDFDV WGAGTTVTVS<br>P | 60<br>120<br>121 |
| SEQ ID NO:116 light chain variable region | DIVMTQSHKF MSTSLGDRVS ITCKASQDVG AAVAWYQQKP GQSPKLLIYW ASTRHTGVPD<br>RFTGGGSGTD FTLTISNVQS EDLTDYFCQQ YINYPLTFGG GTKLEIKR | 60<br>108 |
| SEQ ID NO:117 heavy chain variable region of humanized antibody | QIQLVQSGPE LKKPGETVKI SCKASGYTFT DYSMHWVKQA PGKGLKWMGW INTETGEPTY<br>ADDFKGRFAF SLETSASTAY LQINNLKNED TATYFCANPY YDYVSYYAMD YWGHGTSVTV<br>SS | 60<br>120<br>122 |
| SEQ ID NO:118 heavy chain variable region of humanized antibody | QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY<br>ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV<br>SS | 60<br>120<br>122 |
| SEQ ID NO:119 light chain variable region of humanized antibody | DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD<br>RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK | 60<br>107 |
| SEQ ID NO:120 light chain variable region of humanized antibody | DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD<br>RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK | 60<br>107 |
| SEQ ID NO:121 heavy chain variable region of humanized antibody | EVQLVESGGG LVQPGESLKL SCESNEYEFP SHDMSWVRKT PEKRLELVAA INSDGGSTYY<br>PDTMERRFII SRDNTKKTLY LQMSSLRSED TALYYCARHY DDYYAWFAYW GQGTLVTVSA | 60<br>120 |
| SEQ ID NO:122 heavy chain variable region of humanized antibody | EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY<br>PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS | 60<br>120 |
| SEQ ID NO:123 light chain | DIVLTQSPAS LAVSLGQRAT ISCRASKSVS TSGYSYMHWY QQKPGQPPKL LIYLASNLES<br>GVPARFSGSG SGTDFTLNIH PVEEEDAATY YCQHSRELPL TFGAGTKLEL K | 60<br>111 |

| | | |
|---|---|---|
| variable region of humanized antibody | | |
| SEQ ID NO:124 light chain variable region of humanized antibody | EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES<br>GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K | 60<br>111 |
| SEQ ID NO:125 heavy chain variable region | MYLGLNYVFI VFLLNGVQSE VKLEESGGGL VQPGGSMKLS CAASGFTFSD AWMDWVRQSP<br>EKGLEWVAEI RSKANNHATY YAESVNGRFT ISRDDSKSSV YLQMNSLRAE DTGIYYCTWG<br>EVFYFDYWGQ GTTLTVSS | 60<br>120<br>138 |
| SEQ ID NO:126 light chain variable region | MRPSIQFLGL LLFWLHGAQC DIQMTQSPSS LSASLGGKVT ITCKSSQDIN KYIAWYQHKP<br>GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLLTFGAG<br>TKLELK | 60<br>120<br>126 |

**[001090]** In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain wherein each of the soluble OX40 binding domains lacks a stalk region (which contributes to trimerisation and provides a certain distance to the cell membrane, but is not part of the OX40 binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

**[001091]** In an embodiment, the OX40 agonist is an OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain, (iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein the TNF superfamily cytokine domain is an OX40 binding domain.

**[001092]** In some embodiments, the OX40 agonist is MEDI6383. MEDI6383 is an OX40 agonistic fusion protein and can be prepared as described in U.S. Patent No. 6,312,700, the disclosure of which is incorporated by reference herein.

**[001093]** In an embodiment, the OX40 agonist is an OX40 agonistic scFv antibody comprising any of the foregoing $V_H$ domains linked to any of the foregoing $V_L$ domains.

**[001094]** In an embodiment, the OX40 agonist is Creative Biolabs OX40 agonist monoclonal antibody MOM-18455, commercially available from Creative Biolabs, Inc., Shirley, NY, USA.

**[001095]** In an embodiment, the OX40 agonist is OX40 agonistic antibody clone Ber-ACT35 commercially available from BioLegend, Inc., San Diego, CA, USA.

## I. Optional Cell Viability Analyses

**[001096]** Optionally, a cell viability assay can be performed after the priming first expansion (sometimes referred to as the initial bulk expansion), using standard assays known in the art. Thus, in certain embodiments, the method comprises performing a cell viability assay subsequent to the priming first expansion. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. Other assays for use in testing viability can include but are not limited to the Alamar blue assay; and the MTT assay.

### 1. Cell Counts, Viability, Flow Cytometry

**[001097]** In some embodiments, cell counts and/or viability are measured. The expression of markers such as but not limited CD3, CD4, CD8, and CD56, as well as any other disclosed or described herein, can be measured by flow cytometry with antibodies, for example but not limited to those commercially available from BD Bio-sciences (BD Biosciences, San Jose, CA) using a FACSCanto™ flow cytometer (BD Biosciences). The cells can be counted manually using a disposable c-chip hemocytometer (VWR, Batavia, IL) and viability can be assessed using any method known in the art, including but not limited to trypan blue staining. The cell viability can also be assayed based on USSN 15/863,634, incorporated by reference herein in its entirety. Cell viability can also be assayed based on U.S. Patent Publication No. 2018/0280436 or International Patent Publication No. WO/2018/081473, both of which are incorporate herein in their entireties for all purposes.

**[001098]** In some cases, the bulk TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to REP and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the bulk or REP TIL populations can be subjected to genetic modifications for suitable treatments.

2. Cell Cultures

**[001099]** In an embodiment, a method for expanding TILs, including those discussed above as well as exemplified in Figure 8, in particular, *e.g.*, Figure 8B and/or Figure 8C, may include using about 5,000 mL to about 25,000 mL of cell medium, about 5,000 mL to about 10,000 mL of cell medium, or about 5,800 mL to about 8,700 mL of cell medium. In some embodiments, the media is a serum free medium. In some embodiments, the media in the priming first expansion is serum free. In some embodiments, the media in the second expansion is serum free. In some embodiments, the media in the priming first expansion and the second expansion (also referred to as rapid second expansion) are both serum free. In an embodiment, expanding the number of TILs uses no more than one type of cell culture medium. Any suitable cell culture medium may be used, *e.g.*, AIM-V cell medium (L-glutamine, 50 μM streptomycin sulfate, and 10 μM gentamicin sulfate) cell culture medium (Invitrogen, Carlsbad CA). In this regard, the inventive methods advantageously reduce the amount of medium and the number of types of medium required to expand the number of TIL. In an embodiment, expanding the number of TIL may comprise feeding the cells no more frequently than every third or fourth day. Expanding the number of cells in a gas permeable container simplifies the procedures necessary to expand the number of cells by reducing the feeding frequency necessary to expand the cells.

**[001100]** In an embodiment, the cell culture medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME).

**[001101]** In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable container containing cell medium including IL-2, 1X antigen-presenting feeder cells, and OKT-3 for a duration of about 1 to 8 days, *e.g.*, about 7 days as a priming first expansion, or about 8 days as a priming first expansion; transferring the TILs to a second gas permeable container and expanding the number of TILs in the second gas permeable container containing cell medium including IL-2, 2X antigen-presenting feeder cells, and OKT-3 for a duration of about 7 to 9 days, *e.g.*, about 7 days, about 8 days, or about 9 days.

**[001102]** In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable

container containing cell medium including IL-2, 1X antigen-presenting feeder cells, and OKT-3 for a duration of about 1 to 7 days, *e.g.*, about 7 days as a priming first expansion; transferring the TILs to a second gas permeable container and expanding the number of TILs in the second gas permeable container containing cell medium including IL-2, 2X antigen-presenting feeder cells, and OKT-3 for a duration of about 7 to 9 days, *e.g.*, about 7 days, about 8 days, or about 9 days.

**[001103]** In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable container containing cell medium including IL-2, 1X antigen-presenting feeder cells, and OKT-3 for a duration of about 1 to 7 days, *e.g.*, about 7 days as a priming first expansion; transferring the TILs to a second gas permeable container and expanding the number of TILs in the second gas permeable container containing cell medium including IL-2, 2X antigen-presenting feeder cells, and OKT-3 for a duration of about 7 to 10 days, *e.g.*, about 7 days, about 8 days, about 9 days or about 10 days.

**[001104]** In an embodiment, TILs are expanded in gas-permeable containers. Gas-permeable containers have been used to expand TILs using PBMCs using methods, compositions, and devices known in the art, including those described in U.S. Patent Application Publication No. 2005/0106717 A1, the disclosures of which are incorporated herein by reference. In an embodiment, TILs are expanded in gas-permeable bags. In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the Xuri Cell Expansion System W25 (GE Healthcare). In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the WAVE Bioreactor System, also known as the Xuri Cell Expansion System W5 (GE Healthcare). In an embodiment, the cell expansion system includes a gas permeable cell bag with a volume selected from the group consisting of about 100 mL, about 200 mL, about 300 mL, about 400 mL, about 500 mL, about 600 mL, about 700 mL, about 800 mL, about 900 mL, about 1 L, about 2 L, about 3 L, about 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, and about 10 L.

**[001105]** In an embodiment, TILs can be expanded in G-Rex flasks (commercially available from Wilson Wolf Manufacturing). Such embodiments allow for cell populations to expand from about $5 \times 10^5$ cells/cm$^2$ to between $10 \times 10^6$ and $30 \times 10^6$ cells/cm$^2$. In an embodiment this is without feeding. In an embodiment, this is without feeding so long as medium resides at a height of about 10 cm in the G-Rex flask. In an embodiment this is without feeding but

with the addition of one or more cytokines. In an embodiment, the cytokine can be added as a bolus without any need to mix the cytokine with the medium. Such containers, devices, and methods are known in the art and have been used to expand TILs, and include those described in U.S. Patent Application Publication No. US 2014/0377739A1, International Publication No. WO 2014/210036 A1, U.S. Patent Application Publication No. us 2013/0115617 A1, International Publication No. WO 2013/188427 A1, U.S. Patent Application Publication No. US 2011/0136228 A1, U.S. Patent No. US 8,809,050 B2, International publication No. WO 2011/072088 A2, U.S. Patent Application Publication No. US 2016/0208216 A1, U.S. Patent Application Publication No. US 2012/0244133 A1, International Publication No. WO 2012/129201 A1, U.S. Patent Application Publication No. US 2013/0102075 A1, U.S. Patent No. US 8,956,860 B2, International Publication No. WO 2013/173835 A1, U.S. Patent Application Publication No. US 2015/0175966 A1, the disclosures of which are incorporated herein by reference. Such processes are also described in Jin *et al.*, *J. Immunotherapy,* **2012**, 35:283-292.

### J. Optional Genetic Engineering of TILs

**[001106]** In some embodiments, the expanded TILs of the present invention are further manipulated before, during, or after an expansion step, including during closed, sterile manufacturing processes, each as provided herein, in order to alter protein expression in a transient manner. In some embodiments, the transiently altered protein expression is due to transient gene editing. In some embodiments, the expanded TILs of the present invention are treated with transcription factors (TFs) and/or other molecules capable of transiently altering protein expression in the TILs. In some embodiments, the TFs and/or other molecules that are capable of transiently altering protein expression provide for altered expression of tumor antigens and/or an alteration in the number of tumor antigen-specific T cells in a population of TILs.

**[001107]** In certain embodiments, the method comprises genetically editing a population of TILs. In certain embodiments, the method comprises genetically editing the first population of TILs, the second population of TILs and/or the third population of TILs.

**[001108]** In some embodiments, the present invention includes genetic editing through nucleotide insertion, such as through ribonucleic acid (RNA) insertion, including insertion of messenger RNA (mRNA) or small (or short) interfering RNA (siRNA), into a population of TILs for promotion of the expression of one or more proteins or inhibition of the expression

of one or more proteins, as well as simultaneous combinations of both promotion of one set of proteins with inhibition of another set of proteins.

**[001109]** In some embodiments, the expanded TILs of the present invention undergo transient alteration of protein expression. In some embodiments, the transient alteration of protein expression occurs in the bulk TIL population prior to first expansion, including, for example in the TIL population obtained from for example, Step A as indicated in Figure 8 (particularly Figure 8B and/or Figure 8C). In some embodiments, the transient alteration of protein expression occurs during the first expansion, including, for example in the TIL population expanded in for example, Step B as indicated in Figure 8 (for example Figure 8B and/or Figure 8C). In some embodiments, the transient alteration of protein expression occurs after the first expansion, including, for example in the TIL population in transition between the first and second expansion (e.g. the second population of TILs as described herein), the TIL population obtained from for example, Step B and included in Step C as indicated in Figure 8. In some embodiments, the transient alteration of protein expression occurs in the bulk TIL population prior to second expansion, including, for example in the TIL population obtained from for example, Step C and prior to its expansion in Step D as indicated in Figure 8. In some embodiments, the transient alteration of protein expression occurs during the second expansion, including, for example in the TIL population expanded in for example, Step D as indicated in Figure 8 (e.g. the third population of TILs). In some embodiments, the transient alteration of protein expression occurs after the second expansion, including, for example in the TIL population obtained from the expansion in for example, Step D as indicated in Figure 8.

**[001110]** In an embodiment, a method of transiently altering protein expression in a population of TILs includes the step of electroporation. Electroporation methods are known in the art and are described, *e.g.*, in Tsong, *Biophys. J.* **1991,** *60,* 297-306, and U.S. Patent Application Publication No. 2014/0227237 A1, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of transiently altering protein expression in population of TILs includes the step of calcium phosphate transfection. Calcium phosphate transfection methods (calcium phosphate DNA precipitation, cell surface coating, and endocytosis) are known in the art and are described in Graham and van der Eb, *Virology* **1973,** *52,* 456-467; Wigler, *et al.*, *Proc. Natl. Acad. Sci.* **1979,** *76,* 1373-1376; and Chen and Okayarea, *Mol. Cell. Biol.* **1987,** *7,* 2745-2752; and in U.S. Patent No. 5,593,875, the disclosures of each of which are incorporated by reference herein. In an embodiment, a

method of transiently altering protein expression in a population of TILs includes the step of liposomal transfection. Liposomal transfection methods, such as methods that employ a 1:1 (w/w) liposome formulation of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE) in filtered water, are known in the art and are described in Rose, *et al.*, *Biotechniques* **1991,** *10,* 520-525 and Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA*, **1987,** *84,* 7413-7417 and in U.S. Patent Nos. 5,279,833; 5,908,635; 6,056,938; 6,110,490; 6,534,484; and 7,687,070, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of transiently altering protein expression in a population of TILs includes the step of transfection using methods described in U.S. Patent Nos. 5,766,902; 6,025,337; 6,410,517; 6,475,994; and 7,189,705; the disclosures of each of which are incorporated by reference herein.

**[001111]** In some embodiments, transient alteration of protein expression results in an increase in Stem Memory T cells (TSCMs). TSCMs are early progenitors of antigen-experienced central memory T cells. TSCMs generally display the long-term survival, self-renewal, and multipotency abilities that define stem cells, and are generally desirable for the generation of effective TIL products. TSCM have shown enhanced anti-tumor activity compared with other T cell subsets in mouse models of adoptive cell transfer (Gattinoni *et al.* Nat Med 2009, 2011; Gattinoni, Nature Rev. Cancer, 2012; Cieri *et al.* Blood 2013). In some embodiments, transient alteration of protein expression results in a TIL population with a composition comprising a high proportion of TSCM. In some embodiments, transient alteration of protein expression results in an at least 5%, at least 10%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% increase in TSCM percentage. In some embodiments, transient alteration of protein expression results in an at least a 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, or 10-fold increase in TSCMs in the TIL population. In some embodiments, transient alteration of protein expression results in a TIL population with at least at least 5%, at least 10%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% TSCMs. In some embodiments, transient alteration of protein expression results in a therapeutic TIL population with at least at least 5%, at least 10%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least

45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% TSCMs.

**[001112]** In some embodiments, transient alteration of protein expression results in rejuvenation of antigen-experienced T-cells. In some embodiments, rejuvenation includes, for example, increased proliferation, increased T-cell activation, and/or increased antigen recognition.

**[001113]** In some embodiments, transient alteration of protein expression alters the expression in a large fraction of the T-cells in order to preserve the tumor-derived TCR repertoire. In some embodiments, transient alteration of protein expression does not alter the tumor-derived TCR repertoire. In some embodiments, transient alteration of protein expression maintains the tumor-derived TCR repertoire.

**[001114]** In some embodiments, transient alteration of protein results in altered expression of a particular gene. In some embodiments, the transient alteration of protein expression targets a gene including but not limited to PD-1 (also referred to as PDCD1 or CC279), TGFBR2, CCR4/5, CBLB (CBL-B), CISH, CCRs (chimeric co-stimulatory receptors), IL-2, IL-12, IL-15, IL-21, NOTCH 1/2 ICD, TIM3, LAG3, TIGIT, TGFβ, CCR2, CCR4, CCR5, CXCR1, CXCR2, CSCR3, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL1/CXCL8, CCL22, CCL17, CXCL1/CXCL8, VHL, CD44, PIK3CD, SOCS1, and/or cAMP protein kinase A (PKA). In some embodiments, the transient alteration of protein expression targets a gene selected from the group consisting of PD-1, TGFBR2, CCR4/5, CBLB (CBL-B), CISH, CCRs (chimeric co-stimulatory receptors), IL-2, IL-12, IL-15, IL-21, NOTCH 1/2 ICD, TIM3, LAG3, TIGIT, TGFβ, CCR2, CCR4, CCR5, CXCR1, CXCR2, CSCR3, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL1/CXCL8, CCL22, CCL17, CXCL1/CXCL8, VHL, CD44, PIK3CD, SOCS1, and/or cAMP protein kinase A (PKA). In some embodiments, the transient alteration of protein expression targets PD-1. In some embodiments, the transient alteration of protein expression targets TGFBR2. In some embodiments, the transient alteration of protein expression targets CCR4/5. In some embodiments, the transient alteration of protein expression targets CBLB. In some embodiments, the transient alteration of protein expression targets CISH. In some embodiments, the transient alteration of protein expression targets CCRs (chimeric co-stimulatory receptors). In some embodiments, the transient alteration of protein expression targets IL-2. In some embodiments, the transient alteration of protein expression targets IL-12. In some embodiments, the transient alteration of protein expression targets IL-15. In

some embodiments, the transient alteration of protein expression targets IL-21. In some embodiments, the transient alteration of protein expression targets NOTCH 1/2 ICD. In some embodiments, the transient alteration of protein expression targets TIM3. In some embodiments, the transient alteration of protein expression targets LAG3. In some embodiments, the transient alteration of protein expression targets TIGIT. In some embodiments, the transient alteration of protein expression targets TGFβ. In some embodiments, the transient alteration of protein expression targets CCR1. In some embodiments, the transient alteration of protein expression targets CCR2. In some embodiments, the transient alteration of protein expression targets CCR4. In some embodiments, the transient alteration of protein expression targets CCR5. In some embodiments, the transient alteration of protein expression targets CXCR1. In some embodiments, the transient alteration of protein expression targets CXCR2. In some embodiments, the transient alteration of protein expression targets CSCR3. In some embodiments, the transient alteration of protein expression targets CCL2 (MCP-1). In some embodiments, the transient alteration of protein expression targets CCL3 (MIP-1α). In some embodiments, the transient alteration of protein expression targets CCL4 (MIP1-β). In some embodiments, the transient alteration of protein expression targets CCL5 (RANTES). In some embodiments, the transient alteration of protein expression targets CXCL1. In some embodiments, the transient alteration of protein expression targets CXCL8. In some embodiments, the transient alteration of protein expression targets CCL22. In some embodiments, the transient alteration of protein expression targets CCL17. In some embodiments, the transient alteration of protein expression targets VHL. In some embodiments, the transient alteration of protein expression targets CD44. In some embodiments, the transient alteration of protein expression targets PIK3CD. In some embodiments, the transient alteration of protein expression targets SOCS1. In some embodiments, the transient alteration of protein expression targets cAMP protein kinase A (PKA).

**[001115]** In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of a chemokine receptor. In some embodiments, the chemokine receptor that is overexpressed by transient protein expression includes a receptor with a ligand that includes but is not limited to CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL1, CXCL8, CCL22, and/or CCL17.

**[001116]** In some embodiments, the transient alteration of protein expression results in a decrease and/or reduced expression of PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, TGFβR2, and/or TGFβ (including resulting in, for example, TGFβ pathway blockade). In some embodiments, the transient alteration of protein expression results in a decrease and/or reduced expression of CBLB (CBL-B). In some embodiments, the transient alteration of protein expression results in a decrease and/or reduced expression of CISH.

**[001117]** In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of chemokine receptors in order to, for example, improve TIL trafficking or movement to the tumor site. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of a CCR (chimeric co-stimulatory receptor). In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of a chemokine receptor selected from the group consisting of CCR1, CCR2, CCR4, CCR5, CXCR1, CXCR2, and/or CSCR3.

**[001118]** In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of an interleukin. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of an interleukin selected from the group consisting of IL-2, IL-12, IL-15, and/or IL-21.

**[001119]** In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of NOTCH 1/2 ICD. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of VHL. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of CD44. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of PIK3CD. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of SOCS1,

**[001120]** In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of cAMP protein kinase A (PKA).

**[001121]** In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of a molecule selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of two molecules selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF

(BR3), and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and one molecule selected from the group consisting of LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1, LAG-3, CISH, CBLB, TIM3, and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and one of LAG3, CISH, CBLB, TIM3, and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and LAG3. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and CISH. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and CBLB. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of LAG3 and CISH. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of LAG3 and CBLB. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of CISH and CBLB. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and PD-1. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and LAG3. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and CISH. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and CBLB.

**[001122]** In some embodiments, an adhesion molecule selected from the group consisting of CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, and combinations thereof, is inserted by a gammaretroviral or lentiviral method into the first population of TILs, second population of TILs, or harvested population of TILs (*e.g.*, the expression of the adhesion molecule is increased).

**[001123]** In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of a molecule selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof, and increased and/or enhanced expression of CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, and combinations thereof. In some embodiments, the transient

alteration of protein expression results in decreased and/or reduced expression of a molecule selected from the group consisting of PD-1, LAG3, TIM3, CISH, CBLB, and combinations thereof, and increased and/or enhanced expression of CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, and combinations thereof.

**[001124]** In some embodiments, there is a reduction in expression of about 5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 80%. In some embodiments, there is a reduction in expression of at least about 85%, In some embodiments, there is a reduction in expression of at least about 90%. In some embodiments, there is a reduction in expression of at least about 95%. In some embodiments, there is a reduction in expression of at least about 99%.

**[001125]** In some embodiments, there is an increase in expression of about 5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 80%. In some embodiments, there is an increase in expression of at least about 85%, In some embodiments, there is an increase in expression of at least about 90%. In some embodiments, there is an increase in expression of at least about 95%. In some embodiments, there is an increase in expression of at least about 99%.

**[001126]** In some embodiments, transient alteration of protein expression is induced by treatment of the TILs with transcription factors (TFs) and/or other molecules capable of transiently altering protein expression in the TILs. In some embodiments, the SQZ vector-free microfluidic platform is employed for intracellular delivery of the transcription factors (TFs) and/or other molecules capable of transiently altering protein expression. Such methods demonstrating the ability to deliver proteins, including transcription factors, to a variety of primary human cells, including T cells (Sharei *et al.* PNAS 2013, as well as Sharei *et al.* PLOS ONE 2015 and Greisbeck *et al.* J. Immunology vol. 195, 2015) have been described; see, for example, International Patent Publications WO 2013/059343A1, WO 2017/008063A1, and WO 2017/123663A1, all of which are incorporated by reference herein in their entireties. Such methods as described in International Patent Publications WO 2013/059343A1, WO 2017/008063A1, and WO 2017/123663A1 can be employed with the present invention in order to expose a population of TILs to transcription factors (TFs) and/or other molecules capable of inducing transient protein expression, wherein said TFs and/or other molecules capable of inducing transient protein expression provide for increased expression of tumor antigens and/or an increase in the number of tumor antigen-specific T cells in the population of TILs, thus resulting in reprogramming of the TIL population and an increase in therapeutic efficacy of the reprogrammed TIL population as compared to a non-reprogrammed TIL population. In some embodiments, the reprogramming results in an increased subpopulation of effector T cells and/or central memory T cells relative to the starting or prior population (*i.e.*, prior to reprogramming) population of TILs, as described herein.

**[001127]** In some embodiments, the transcription factor (TF) includes but is not limited to TCF-1, NOTCH 1/2 ICD, and/or MYB. In some embodiments, the transcription factor (TF) is TCF-1. In some embodiments, the transcription factor (TF) is NOTCH 1/2 ICD. In some embodiments, the transcription factor (TF) is MYB. In some embodiments, the transcription factor (TF) is administered with induced pluripotent stem cell culture (iPSC), such as the commercially available KNOCKOUT Serum Replacement (Gibco/ThermoFisher), to induce additional TIL reprogramming. In some embodiments, the transcription factor (TF) is administered with an iPSC cocktail to induce additional TIL reprogramming. In some embodiments, the transcription factor (TF) is administered without an iPSC cocktail. In some embodiments, reprogramming results in an increase in the percentage of TSCMs. In some embodiments, reprogramming results in an increase in the percentage of TSCMs by about

5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% TSCMs.

**[001128]** In some embodiments, a method of transient altering protein expression, as described above, may be combined with a method of genetically modifying a population of TILs includes the step of stable incorporation of genes for production of one or more proteins. In certain embodiments, the method comprises a step of genetically modifying a population of TILs. In certain embodiments, the method comprises genetically modifying the first population of TILs, the second population of TILs and/or the third population of TILs. In an embodiment, a method of genetically modifying a population of TILs includes the step of retroviral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of lentiviral transduction. Lentiviral transduction systems are known in the art and are described, *e.g.*, in Levine, *et al.*, *Proc. Nat'l Acad. Sci.* **2006,** *103,* 17372-77; Zufferey, *et al., Nat. Biotechnol.* **1997,** *15,* 871-75; Dull, *et al.*, *J. Virology* **1998,** *72,* 8463-71, and U.S. Patent No. 6,627,442, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of gamma-retroviral transduction. Gamma-retroviral transduction systems are known in the art and are described, *e.g.*, Cepko and Pear, *Cur. Prot. Mol. Biol.* **1996,** 9.9.1-9.9.16, the disclosure of which is incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of transposon-mediated gene transfer. Transposon-mediated gene transfer systems are known in the art and include systems wherein the transposase is provided as DNA expression vector or as an expressible RNA or a protein such that long-term expression of the transposase does not occur in the transgenic cells, for example, a transposase provided as an mRNA (*e.g.*, an mRNA comprising a cap and poly-A tail). Suitable transposon-mediated gene transfer systems, including the salmonid-type Tel-like transposase (SB or Sleeping Beauty transposase), such as SB10, SB11, and SB100x, and engineered enzymes with increased enzymatic activity, are described in, *e.g.*, Hackett, *et al.*, *Mol. Therapy* **2010,** *18,* 674-83 and U.S. Patent No. 6,489,458, the disclosures of each of which are incorporated by reference herein.

**[001129]** In some embodiments, transient alteration of protein expression is a reduction in expression induced by self-delivering RNA interference (sdRNA), which is a chemically-synthesized asymmetric siRNA duplex with a high percentage of 2'-OH substitutions

(typically fluorine or -$OCH_3$) which comprises a 20-nucleotide antisense (guide) strand and a 13 to 15 base sense (passenger) strand conjugated to cholesterol at its 3' end using a tetraethylenglycol (TEG) linker. In some embodiments, the method comprises transient alteration of protein expression in a population of TILs, comprising the use of self-delivering RNA interference (sdRNA), which is a chemically-synthesized asymmetric siRNA duplex with a high percentage of 2'-OH substitutions (typically fluorine or -$OCH_3$) which comprises a 20-nucleotide antisense (guide) strand and a 13 to 15 base sense (passenger) strand conjugated to cholesterol at its 3' end using a tetraethylenglycol (TEG) linker. Methods of using sdRNA have been described in Khvorova and Watts, *Nat. Biotechnol.* **2017,** *35,* 238–248; Byrne, *et al.*, *J. Ocul. Pharmacol. Ther.* **2013,** *29,* 855-864; and Ligtenberg, *et al.*, *Mol. Therapy,* **2018,** in press, the disclosures of which are incorporated by reference herein. In an embodiment, delivery of sdRNA to a TIL population is accomplished without use of electroporation, SQZ, or other methods, instead using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of 1 μM/10,000 TILs in medium. In certain embodiments, the method comprises delivery sdRNA to a TILs population comprising exposing the TILs population to sdRNA at a concentration of 1 μM/10,000 TILs in medium for a period of between 1 to 3 days. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of 10 μM/10,000 TILs in medium. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of 50 μM/10,000 TILs in medium. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of between 0.1 μM/10,000 TILs and 50 μM/10,000 TILs in medium. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of between 0.1 μM/10,000 TILs and 50 μM/10,000 TILs in medium, wherein the exposure to sdRNA is performed two, three, four, or five times by addition of fresh sdRNA to the media. Other suitable processes are described, for example, in U.S. Patent Application Publication No. US 2011/0039914 A1, US 2013/0131141 A1, and US 2013/0131142 A1, and U.S. Patent No. 9,080,171, the disclosures of which are incorporated by reference herein.

**[001130]** In some embodiments, sdRNA is inserted into a population of TILs during manufacturing. In some embodiments, the sdRNA encodes RNA that interferes with

NOTCH 1/2 ICD, PD-1, CTLA-4 TIM-3, LAG-3, TIGIT, TGFβ, TGFBR2, cAMP protein kinase A (PKA), BAFF BR3, CISH, and/or CBLB. In some embodiments, the reduction in expression is determined based on a percentage of gene silencing, for example, as assessed by flow cytometry and/or qPCR. In some embodiments, there is a reduction in expression of about 5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 80%. In some embodiments, there is a reduction in expression of at least about 85%, In some embodiments, there is a reduction in expression of at least about 90%. In some embodiments, there is a reduction in expression of at least about 95%. In some embodiments, there is a reduction in expression of at least about 99%.

**[001131]** The self-deliverable RNAi technology based on the chemical modification of siRNAs can be employed with the methods of the present invention to successfully deliver the sdRNAs to the TILs as described herein. The combination of backbone modifications with asymmetric siRNA structure and a hydrophobic ligand (*see*, for eample, Ligtenberg, *et al.*, *Mol. Therapy,* **2018** and US20160304873) allow sdRNAs to penetrate cultured mammalian cells without additional formulations and methods by simple addition to the culture media, capitalizing on the nuclease stability of sdRNAs. This stability allows the support of constant levels of RNAi-mediated reduction of target gene activity simply by maintaining the active concentration of sdRNA in the media. While not being bound by theory, the backbone stabilization of sdRNA provides for extended reduction in gene expression effects which can last for months in non-dividing cells.

**[001132]** In some embodiments, over 95% transfection efficiency of TILs and a reduction in expression of the target by various specific sdRNA occurs. In some embodiments, sdRNAs containing several unmodified ribose residues were replaced with fully modified sequences to increase potency and/or the longevity of RNAi effect. In some embodiments, a reduction in expression effect is maintained for 12 hours, 24 hours, 36 hours, 48 hours, 5 days, 6 days, 7

dyas, or 8 days or more. In some embodiments, the reduction in expression effect decreases at 10 days or more post sdRNA treatment of the TILs. In some embodiments, more than 70% reduction in expression of the target expression is maintained. In some embodiments, more than 70% reduction in expression of the target expression is maintained TILs. In some embodiments, a reduction in expression in the PD-1/PD-L1 pathway allows for the TILs to exhibit a more potent in vivo effect, which is in some embodiments, due to the avoidance of the suppressive effects of the PD-1/PD-L1 pathway. In some embodiments, a reduction in expression of PD-1 by sdRNA results in an increase TIL proliferation.

**[001133]** Small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing RNA, is a double stranded RNA molecule, generally 19-25 base pairs in length. siRNA is used in RNA interference (RNAi), where it interferes with expression of specific genes with complementary nucleotide sequences.

**[001134]** Double stranded DNA (dsRNA) can be generally used to define any molecule comprising a pair of complementary strands of RNA, generally a sense (passenger) and antisense (guide) strands, and may include single-stranded overhang regions. The term dsRNA, contrasted with siRNA, generally refers to a precursor molecule that includes the sequence of an siRNA molecule which is released from the larger dsRNA molecule by the action of cleavage enzyme systems, including Dicer.

**[001135]** sdRNA (self-deliverable RNA) are a new class of covalently modified RNAi compounds that do not require a delivery vehicle to enter cells and have improved pharmacology compared to traditional siRNAs. "Self-deliverable RNA" or "sdRNA" is a hydrophobically modified RNA interfering-antisense hybrid, demonstrated to be highly efficacious *in vitro* in primary cells and in vivo upon local administration. Robust uptake and/or silencing without toxicity has been demonstrated. sdRNAs are generally asymmetric chemically modified nucleic acid molecules with minimal double stranded regions. sdRNA molecules typically contain single stranded regions and double stranded regions, and can contain a variety of chemical modifications within both the single stranded and double stranded regions of the molecule. Additionally, the sdRNA molecules can be attached to a hydrophobic conjugate such as a conventional and advanced sterol-type molecule, as described herein. sdRNAs and associated methods for making such sdRNAs have also been described extensively in, for example, US20160304873, WO2010033246, WO2017070151, WO2009102427, WO2011119887, WO2010033247A2, WO2009045457, WO2011119852, all of which are incorporated by reference herein in their entireties for all purposes. To

optimize sdRNA structure, chemistry, targeting position, sequence preferences, and the like, a proprietary algorithm has been developed and utilized for sdRNA potency prediction (*see*, for example, US 20160304873). Based on these analyses, functional sdRNA sequences have been generally defined as having over 70% reduction in expression at 1 μM concentration, with a probability over 40%.

**[001136]** In some embodiments, the sdRNA sequences used in the invention exhibit a 70% reduction in expression of the target gene. In some embodiments, the sdRNA sequences used in the invention exhibit a 75% reduction in expression of the target gene.
In some embodiments, the sdRNA sequences used in the invention exhibit an 80% reduction in expression of the target gene. In some embodiments, the sdRNA sequences used in the invention exhibit an 85% reduction in expression of the target gene. In some embodiments, the sdRNA sequences used in the invention exhibit a 90% reduction in expression of the target gene. In some embodiments, the sdRNA sequences used in the invention exhibit a 95% reduction in expression of the target gene. In some embodiments, the sdRNA sequences used in the invention exhibit a 99% reduction in expression of the target gene. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 0.25 μM to about 4 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 0.25 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 0.5 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 0.75 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 1.0 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 1.25 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 1.5 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 1.75 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 2.0 μM. In some embodiments, the sdRNA sequences used in the

invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 2.25 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 2.5 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 2.75 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 3.0 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 3.25 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 3.5 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 3.75 μM. In some embodiments, the sdRNA sequences used in the invention exhibit a reduction in expression of the target gene when delivered at a concentration of about 4.0 μM.

**[001137]** In some emodiments, the oligonucleotide agents comprise one or more modification to increase stability and/or effectiveness of the therapeutic agent, and to effect efficient delivery of the oligonucleotide to the cells or tissue to be treated. Such modifications can include a 2'-O-methyl modification, a 2'-O-Fluro modification, a diphosphorothioate modification, 2' F modified nucleotide, a2'-O-methyl modified and/or a 2'deoxy nucleotide. In some embodiments, the oligonucleotide is modified to include one or more hydrophobic modifications including, for example, sterol, cholesterol, vitamin D, naphtyl, isobutyl, benzyl, indol, tryptophane, and/or phenyl. In an additional particular embodiment, chemically modified nucleotides are combination of phosphorothioates, 2'-O-methyl, 2'deoxy, hydrophobic modifications and phosphorothioates. In some embodiments, the sugars can be modified and modified sugars can include but are not limited to D-ribose, 2'-O-alkyl (including 2'-O-methyl and 2'-0-ethyl), i.e., 2'-alkoxy, 2'-amino, 2'-S-alkyl, 2'-halo (including 2'-fluoro), T- methoxyethoxy, 2'-allyloxy ($-OCH_2CH=CH_2$), 2'-propargyl, 2'-propyl, ethynyl, ethenyl, propenyl, and cyano and the like. In one embodiment, the sugar moiety can be a hexose and incorporated into an oligonucleotide as described (Augustyns, K., et al., Nucl. Acids. Res. 18:4711 (1992)).

**[001138]** In some embodiments, the double-stranded oligonucleotide of the invention is double-stranded over its entire length, *i.e.*, with no overhanging single-stranded sequence at either end of the molecule, i.e., is blunt-ended. In some embodiments, the individual nucleic acid molecules can be of different lengths. In other words, a double-stranded oligonucleotide of the invention is not double-stranded over its entire length. For instance, when two separate nucleic acid molecules are used, one of the molecules, e.g., the first molecule comprising an antisense sequence, can be longer than the second molecule hybridizing thereto (leaving a portion of the molecule single-stranded). In some embodiments, when a single nucleic acid molecule is used a portion of the molecule at either end can remain single-stranded.

**[001139]** In some embodiments, a double-stranded oligonucleotide of the invention contains mismatches and/or loops or bulges, but is double-stranded over at least about 70% of the length of the oligonucleotide. In some embodiments, a double-stranded oligonucleotide of the invention is double-stranded over at least about 80% of the length of the oligonucleotide. In another embodiment, a double-stranded oligonucleotide of the invention is double-stranded over at least about 90%-95% of the length of the oligonucleotide. In some embodiments, a double-stranded oligonucleotide of the invention is double-stranded over at least about 96%-98% of the length of the oligonucleotide. In some embodiments, the double-stranded oligonucleotide of the invention contains at least or up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mismatches.

**[001140]** In some embodiments, the oligonucleotide can be substantially protected from nucleases e.g., by modifying the 3' or 5' linkages (e.g., U.S. Pat. No. 5,849,902 and WO 98/13526). For example, oligonucleotides can be made resistant by the inclusion of a "blocking group." The term "blocking group" as used herein refers to substituents (e.g., other than OH groups) that can be attached to oligonucleotides or nucleomonomers, either as protecting groups or coupling groups for synthesis (e.g., FITC, propyl ($CH_2$-$CH_2$-$CH_3$), glycol (-0-$CH_2$-$CH_2$-O-) phosphate ($PO_3^{2"}$), hydrogen phosphonate, or phosphoramidite). "Blocking groups" can also include "end blocking groups" or "exonuclease blocking groups" which protect the 5' and 3' termini of the oligonucleotide, including modified nucleotides and non-nucleotide exonuclease resistant structures.

**[001141]** In some embodiments, at least a portion of the contiguous polynucleotides within the sdRNA are linked by a substitute linkage, *e.g.*, a phosphorothioate linkage.

**[001142]** In some embodiments, chemical modification can lead to at least a 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 enhancements in cellular uptake. In some embodiments, at least one of the C or U residues includes a hydrophobic modification. In some embodiments, a plurality of Cs and Us contain a hydrophobic modification. In some embodiments, at least 10%, 15%, 20%, 30%, 40%, 50%, 55%, 60% 65%, 70%, 75%, 80%, 85%, 90% or at least 95% of the Cs and Us can contain a hydrophobic modification. In some embodiments, all of the Cs and Us contain a hydrophobic modification.

**[001143]** In some embodiments, the sdRNA or sd-rxRNAs exhibit enhanced endosomal release of sd-rxRNA molecules through the incorporation of protonatable amines. In some embodiments, protonatable amines are incorporated in the sense strand (in the part of the molecule which is discarded after RISC loading). In some embodiments, the sdRNA compounds of the invention comprise an asymmetric compound comprising a duplex region (required for efficient RISC entry of 10-15 bases long) and single stranded region of 4-12 nucleotides long; with a 13 nucleotide duplex. In some embodiments, a 6 nucleotide single stranded region is employed. In some embodiments, the single stranded region of the sdRNA comprises 2-12 phosphorothioate intemucleotide linkages (referred to as phosphorothioate modifications). In some embodiments, 6-8 phosphorothioate intemucleotide linkages are employed. In some embodiments, the sdRNA compounds of the invention also include a unique chemical modification pattern, which provides stability and is compatible with RISC entry.

**[001144]** The guide strand, for example, may also be modified by any chemical modification which confirms stability without interfering with RISC entry. In some embodiments, the chemical modification pattern in the guide strand includes the majority of C and U nucleotides being 2' F modified and the 5 ' end being phosphorylated.

**[001145]** In some embodiments, at least 30% of the nucleotides in the sdRNA or sd-rxRNA are modified. In some embodiments, at least 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the

nucleotides in the sdRNA or sd-rxRNA are modified. In some embodiments, 100% of the nucleotides in the sdRNA or sd-rxRNA are modified.

**[001146]** In some embodiments, the sdRNA molecules have minimal double stranded regions. In some embodiments the region of the molecule that is double stranded ranges from 8-15 nucleotides long. In some embodiments, the region of the molecule that is double stranded is 8, 9, 10, 11, 12, 13, 14 or 15 nucleotides long. In some embodiments the double stranded region is 13 nucleotides long. There can be 100% complementarity between the guide and passenger strands, or there may be one or more mismatches between the guide and passenger strands. In some embodiments, on one end of the double stranded molecule, the molecule is either blunt-ended or has a one-nucleotide overhang. The single stranded region of the molecule is in some embodiments between 4-12 nucleotides long. In some embodiments, the single stranded region can be 4, 5, 6, 7, 8, 9, 10, 11 or 12 nucleotides long. In some embodiments, the single stranded region can also be less than 4 or greater than 12 nucleotides long. In certain embodiments, the single stranded region is 6 or 7 nucleotides long.

**[001147]** In some embodiments, the sdRNA molecules have increased stability. In some instances, a chemically modified sdRNA or sd-rxRNA molecule has a half-life in media that is longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or more than 24 hours, including any intermediate values. In some embodiments, the sd-rxRNA has a half-life in media that is longer than 12 hours.

**[001148]** In some embodiments, the sdRNA is optimized for increased potency and/or reduced toxicity. In some embodiments, nucleotide length of the guide and/or passenger strand, and/or the number of phosphorothioate modifications in the guide and/or passenger strand, can in some aspects influence potency of the RNA molecule, while replacing 2'-fluoro (2'F) modifications with 2'-0-methyl (2'OMe) modifications can in some aspects influence toxicity of the molecule. In some embodiments, reduction in 2'F content of a molecule is predicted to reduce toxicity of the molecule. In some embodiments, the number of phosphorothioate modifications in an RNA molecule can influence the uptake of the molecule into a cell, for example the efficiency of passive uptake of the molecule into a cell. In some embodiments, the sdRNA has no 2'F modification and yet are characterized by equal efficacy in cellular uptake and tissue penetration.

**[001149]** In some embodiments, a guide strand is approximately 18-19 nucleotides in length and has approximately 2-14 phosphate modifications. For example, a guide strand can contain 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or more than 14 nucleotides that are phosphate-modified. The guide strand may contain one or more modifications that confer increased stability without interfering with RISC entry. The phosphate modified nucleotides, such as phosphorothioate modified nucleotides, can be at the 3' end, 5' end or spread throughout the guide strand. In some embodiments, the 3' terminal 10 nucleotides of the guide strand contain 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 phosphorothioate modified nucleotides. The guide strand can also contain 2'F and/or 2'OMe modifications, which can be located throughout the molecule. In some embodiments, the nucleotide in position one of the guide strand (the nucleotide in the most 5' position of the guide strand) is 2'OMe modified and/or phosphorylated. C and U nucleotides within the guide strand can be 2'F modified. For example, C and U nucleotides in positions 2-10 of a 19 nt guide strand (or corresponding positions in a guide strand of a different length) can be 2'F modified. C and U nucleotides within the guide strand can also be 2'OMe modified. For example, C and U nucleotides in positions 11-18 of a 19 nt guide strand (or corresponding positions in a guide strand of a different length) can be 2'OMe modified. In some embodiments, the nucleotide at the most 3' end of the guide strand is unmodified. In certain embodiments, the majority of Cs and Us within the guide strand are 2'F modified and the 5' end of the guide strand is phosphorylated. In other embodiments, position 1 and the Cs or Us in positions 11-18 are 2'OMe modified and the 5' end of the guide strand is phosphorylated. In other embodiments, position 1 and the Cs or Us in positions 11-18 are 2'OMe modified, the 5' end of the guide strand is phosphorylated, and the Cs or Us in position 2-10 are 2'F modified.

**[001150]** The self-deliverable RNAi technology provides a method of directly transfecting cells with the RNAi agent, without the need for additional formulations or techniques. The ability to transfect hard-to-transfect cell lines, high *in vivo* activity, and simplicity of use, are characteristics of the compositions and methods that present significant functional advantages over traditional siRNA-based techniques, and as such, the sdRNA methods are employed in several embodiments related to the methods of reduction in expression of the target gene in the TILs of the present invention. The sdRNAi methods allows direct delivery of chemically synthesized compounds to a wide range of primary cells and tissues, both *ex-vivo* and *in vivo*. The sdRNAs described in some embodiments of the invention herein are commercially available from Advirna LLC, Worcester, MA, USA.

**[001151]** The sdRNA are formed as hydrophobically-modified siRNA-antisense oligonucleotide hybrid structures, and are disclosed, for example in Byrne et al., December 2013, J. Ocular Pharmacology and Therapeutics, 29(10): 855-864, incorporated by reference herein in its entirety.

**[001152]** In some embodiments, the sdRNA oligonucleotides can be delivered to the TILs described herein using sterile electroporation. In certain embodiments, the method comprises sterile electroporation of a population of TILs to deliver sdRNA oligonucleotides.

**[001153]** In some embodiments, the oligonucleotides can be delivered to the cells in combination with a transmembrane delivery system. In some embodimets, this transmembrane delivery system comprises lipids, viral vectors, and the like. In some embodiments, the oligonucleotide agent is a self-delivery RNAi agent, that does not require any delivery agents. In certain embodiments, the method comprises use of a transmembrane delivery system to deliver sdRNA oligonucleotides to a population of TILs.

**[001154]** Oligonucleotides and oligonucleotide compositions are contacted with (e.g., brought into contact with, also referred to herein as administered or delivered to) and taken up by TILs described herein, including through passive uptake by TILs. The sdRNA can be added to the TILs as described herein during the first expansion, for example Step B, after the first expansion, for example, during Step C, before or during the second expansion, for example before or during Step D, after Step D and before harvest in Step E, during or after harvest in Step F, before or during final formulation and/or transfer to infusion Bag in Step F, as well as before any optional cryopreservation step in Step F. Mroeover, sdRNA can be added after thawing from any cryopreservation step in Step F. In an embodiment, one or more sdRNAs targeting genes as described herein, including PD-1, LAG-3, TIM-3, CISH, and CBLB, may be added to cell culture media comprising TILs and other agents at concentrations selected from the group consisting of 100 nM to 20 mM, 200 nM to 10 mM, 500 nm to 1 mM, 1 μM to 100 μM, and 1 μM to 100 μM. In an embodiment, one or more sdRNAs targeting genes as described herein, including PD-1, LAG-3, TIM-3, CISH, and CBLB, may be added to cell culture media comprising TILs and other agents at amounts selected from the group consisting of 0.1 μM sdRNA/10,000 TILs/100 μL media, 0.5 μM sdRNA/10,000 TILs /100 μL media, 0.75 μM sdRNA/10,000 TILs /100 μL media, 1 μM sdRNA/10,000 TILs /100 μL media, 1.25 μM sdRNA/10,000 TILs /100 μL media, 1.5 μM sdRNA/10,000 TILs /100 μL media, 2 μM sdRNA/10,000 TILs /100 μL media, 5 μM sdRNA/10,000 TILs /100 μL media, or 10 μM sdRNA/10,000 TILs /100 μL media. In an embodiment, one or more sdRNAs

targeting genes as described herein, including PD-1, LAG-3, TIM-3, CISH, and CBLB, may be added to TIL cultures during the pre-REP or REP stages twice a day, once a day, every two days, every three days, every four days, every five days, every six days, or every seven days.

**[001155]** Oligonucleotide compositions of the invention, including sdRNA, can be contacted with TILs as described herein during the expansion process, for example by dissolving sdRNA at high concentrations in cell culture media and allowing sufficient time for passive uptake to occur. In certain embodiments, the method of the present invention comprises contacting a population of TILs with an oligonucleotide composition as described herein. In certain embodiments, the method comprises dissolving an oligonucleotide e.g. sdRNA in a cell culture media and contacting the cell culture media with a population of TILs. The TILs may be a first population, a second population and/or a third population as described herein.

**[001156]** In some embodiments, delivery of oligonucleotides into cells can be enhanced by suitable art recognized methods including calcium phosphate, DMSO, glycerol or dextran, electroporation, or by transfection, e.g., using cationic, anionic, or neutral lipid compositions or liposomes using methods known in the art (*see*, e.g., WO 90/14074; WO 91/16024; WO 91/17424; U.S. Pat. No. 4,897,355; Bergan et a 1993. Nucleic Acids Research. 21 :3567).

**[001157]** In some embodiments, more than one sdRNA is used to reduce expression of a target gene. In some embodiments, one or more of PD-1, TIM-3, CBLB, LAG3 and/or CISH targeting sdRNAs are used together. In some embodiments, a PD-1 sdRNA is used with one or more of TIM-3, CBLB, LAG3 and/or CISH in order to reduce expression of more than one gene target. In some embodiments, a LAG3 sdRNA is used in combination with a CISH targeting sdRNA to reduce gene expression of both targets. In some embodiments, the sdRNAs targeting one or more of PD-1, TIM-3, CBLB, LAG3 and/or CISH herein are commercially available from Advirna LLC, Worcester, MA, USA.

**[001158]** In some embodiments, the sdRNA targets a gene selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In some embodiments, the sdRNA targets a gene selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In some embodiments, one sdRNA targets PD-1 and another sdRNA targets a gene selected from the group consisting of LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In

some embodiments, the sdRNA targets a gene selected from PD-1, LAG-3, CISH, CBLB, TIM3, and combinations thereof. In some embodiments, the sdRNA targets a gene selected from PD-1 and one of LAG3, CISH, CBLB, TIM3, and combinations thereof. In some embodiments, one sdRNA targets PD-1 and one sdRNA targets LAG3. In some embodiments, one sdRNA targets PD-1 and one sdRNA targets CISH. In some embodiments, one sdRNA targets PD-1 and one sdRNA targets CBLB. In some embodiments, one sdRNA targets LAG3 and one sdRNA targets CISH. In some embodiments, one sdRNA targets LAG3 and one sdRNA targets CBLB. In some embodiments, one sdRNA targets CISH and one sdRNA targets CBLB. In some embodiments, one sdRNA targets TIM3 and one sdRNA targets PD-1. In some embodiments, one sdRNA targets TIM3 and one sdRNA targets LAG3. In some embodiments, one sdRNA targets TIM3 and one sdRNA targets CISH. In some embodiments, one sdRNA targets TIM3 and one sdRNA targets CBLB.

**[001159]** As discussed above, embodiments of the present invention provide tumor infiltrating lymphocytes (TILs) that have been genetically modified via gene-editing to enhance their therapeutic effect. Embodiments of the present invention embrace genetic editing through nucleotide insertion (RNA or DNA) into a population of TILs for both promotion of the expression of one or more proteins and inhibition of the expression of one or more proteins, as well as combinations thereof. Embodiments of the present invention also provide methods for expanding TILs into a therapeutic population, wherein the methods comprise gene-editing the TILs. There are several gene-editing technologies that may be used to genetically modify a population of TILs, which are suitable for use in accordance with the present invention.

**[001160]** In some embodiments, the method comprises a method of genetically modifying a population of TILs which include the step of stable incorporation of genes for production of one or more proteins. In an embodiment, a method of genetically modifying a population of TILs includes the step of retroviral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of lentiviral transduction. Lentiviral transduction systems are known in the art and are described, *e.g.*, in Levine, *et al.*, *Proc. Nat'l Acad. Sci.* **2006,** *103,* 17372-77; Zufferey, *et al., Nat. Biotechnol.* **1997,** *15,* 871-75; Dull, *et al.*, *J. Virology* **1998,** *72*, 8463-71, and U.S. Patent No. 6,627,442, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of gamma-retroviral transduction. Gamma-

retroviral transduction systems are known in the art and are described, *e.g.*, Cepko and Pear, *Cur. Prot. Mol. Biol.* **1996,** 9.9.1-9.9.16, the disclosure of which is incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of transposon-mediated gene transfer. Transposon-mediated gene transfer systems are known in the art and include systems wherein the transposase is provided as DNA expression vector or as an expressible RNA or a protein such that long-term expression of the transposase does not occur in the transgenic cells, for example, a transposase provided as an mRNA (*e.g.*, an mRNA comprising a cap and poly-A tail). Suitable transposon-mediated gene transfer systems, including the salmonid-type Tel-like transposase (SB or Sleeping Beauty transposase), such as SB10, SB11, and SB100x, and engineered enzymes with increased enzymatic activity, are described in, *e.g.*, Hackett, *et al.*, *Mol. Therapy* **2010,** *18,* 674-83 and U.S. Patent No. 6,489,458, the disclosures of each of which are incorporated by reference herein.

**[001161]** In an embodiment, the method comprises a method of genetically modifying a population of TILs e.g. a first population, a second population and/or a third population as described herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of stable incorporation of genes for production or inhibition (e.g., silencing) of one ore more proteins. In an embodiment, a method of genetically modifying a population of TILs includes the step of electroporation. Electroporation methods are known in the art and are described, *e.g.*, in Tsong, *Biophys. J.* **1991,** *60,* 297-306, and U.S. Patent Application Publication No. 2014/0227237 A1, the disclosures of each of which are incorporated by reference herein. Other electroporation methods known in the art, such as those described in U.S. Patent Nos. 5,019,034; 5,128,257; 5,137,817; 5,173,158; 5,232,856; 5,273,525; 5,304,120; 5,318,514; 6,010,613 and 6,078,490, the disclosures of which are incorporated by reference herein, may be used. In an embodiment, the electroporation method is a sterile electroporation method. In an embodiment, the electroporation method is a pulsed electroporation method. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein the sequence of at least three DC electrical pulses has one, two, or three of the following characteristics: (1) at least two of the at least three pulses

differ from each other in pulse amplitude; (2) at least two of the at least three pulses differ from each other in pulse width; and (3) a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein at least two of the at least three pulses differ from each other in pulse amplitude. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein at least two of the at least three pulses differ from each other in pulse width. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to induce pore formation in the TILs, comprising the step of applying a sequence of at least three DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to TILs, wherein the sequence of at least three DC electrical pulses has one, two, or three of the following characteristics: (1) at least two of the at least three pulses differ from each other in pulse amplitude; (2) at least two of the at least three pulses differ from each other in pulse width; and (3) a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses, such that induced pores are sustained for a relatively long period of time, and such that viability of the TILs is maintained. In an embodiment, a method of genetically modifying a population of TILs includes the step of calcium phosphate transfection. Calcium phosphate transfection methods (calcium phosphate DNA

precipitation, cell surface coating, and endocytosis) are known in the art and are described in Graham and van der Eb, *Virology* **1973,** *52,* 456-467; Wigler, *et al.*, *Proc. Natl. Acad. Sci.* **1979,** *76,* 1373-1376; and Chen and Okayarea, *Mol. Cell. Biol.* **1987,** *7,* 2745-2752; and in U.S. Patent No. 5,593,875, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of liposomal transfection. Liposomal transfection methods, such as methods that employ a 1:1 (w/w) liposome formulation of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE) in filtered water, are known in the art and are described in Rose, *et al.*, *Biotechniques* **1991,** *10,* 520-525 and Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA*, **1987,** *84,* 7413-7417 and in U.S. Patent Nos. 5,279,833; 5,908,635; 6,056,938; 6,110,490; 6,534,484; and 7,687,070, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of transfection using methods described in U.S. Patent Nos. 5,766,902; 6,025,337; 6,410,517; 6,475,994; and 7,189,705; the disclosures of each of which are incorporated by reference herein. The TILs may be a first population, a second population and/or a third population of TILs as described herein.

**[001162]** According to an embodiment, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at one or more immune checkpoint genes. Such programmable nucleases enable precise genome editing by introducing breaks at specific genomic loci, *i.e.,* they rely on the recognition of a specific DNA sequence within the genome to target a nuclease domain to this location and mediate the generation of a double-strand break at the target sequence. A double-strand break in the DNA subsequently recruits endogenous repair machinery to the break site to mediate genome editing by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). Thus, the repair of the break can result in the introduction of insertion/deletion mutations that disrupt (*e.g.*, silence, repress, or enhance) the target gene product.

**[001163]** Major classes of nucleases that have been developed to enable site-specific genomic editing include zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and CRISPR-associated nucleases (e.g., CRISPR/Cas9). These nuclease systems can be broadly classified into two categories based on their mode of DNA recognition: ZFNs and TALENs achieve specific DNA binding via protein-DNA interactions, whereas CRISPR

systems, such as Cas9, are targeted to specific DNA sequences by a short RNA guide molecule that base-pairs directly with the target DNA and by protein-DNA interactions. *See, e.g.,* Cox *et al.*, *Nature Medicine,* 2015, Vol. 21, No. 2.

**[001164]** Non-limiting examples of gene-editing methods that may be used in accordance with TIL expansion methods of the present invention include CRISPR methods, TALE methods, and ZFN methods, which are described in more detail below. According to an embodiment, a method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (e.g., GEN 3 process) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by one or more of a CRISPR method, a TALE method or a ZFN method, in order to generate TILs that can provide an enhanced therapeutic effect. According to an embodiment, gene-edited TILs can be evaluated for an improved therapeutic effect by comparing them to non-modified TILs *in vitro, e.g.,* by evaluating *in vitro* effector function, cytokine profiles, etc. compared to unmodified TILs. In certain embodiments, the method comprises gene editing a population of TILs using CRISPR, TALE and/ or ZFN methods.

**[001165]** In some embodiments of the present invention, electroporation is used for delivery of a gene editing system, such as CRISPR, TALEN, and ZFN systems. In some embodiments of the present invention, the electroporation system is a flow electroporation system. An example of a suitable flow electroporation system suitable for use with some embodiments of the present invention is the commercially-available MaxCyte STX system. There are several alternative commercially-available electroporation instruments which may be suitable for use with the present invention, such as the AgilePulse system or ECM 830 available from BTX-Harvard Apparatus, Cellaxess Elektra (Cellectricon), Nucleofector (Lonza/Amaxa), GenePulser MXcell (BIORAD), iPorator-96 (Primax) or siPORTer96 (Ambion). In some embodiments of the present invention, the electroporation system forms a closed, sterile system with the remainder of the TIL expansion method. In some embodiments of the present invention, the electroporation system is a pulsed electroporation system as described herein, and forms a closed, sterile system with the remainder of the TIL expansion method.

**[001166]** A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (e.g., process GEN 3) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein

the method further comprises gene-editing at least a portion of the TILs by a CRISPR method (*e.g.,* CRISPR/Cas9 or CRISPR/Cpf1). According to particular embodiments, the use of a CRISPR method during the TIL expansion process causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a CRISPR method during the TIL expansion process causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

**[001167]** CRISPR stands for "Clustered Regularly Interspaced Short Palindromic Repeats." A method of using a CRISPR system for gene editing is also referred to herein as a CRISPR method. There are three types of CRISPR systems which incorporate RNAs and Cas proteins, and which may be used in accordance with the present invention: Types I, II, and III. The Type II CRISPR (exemplified by Cas9) is one of the most well-characterized systems.

**[001168]** CRISPR technology was adapted from the natural defense mechanisms of bacteria and archaea (the domain of single-celled microorganisms). These organisms use CRISPR-derived RNA and various Cas proteins, including Cas9, to foil attacks by viruses and other foreign bodies by chopping up and destroying the DNA of a foreign invader. A CRISPR is a specialized region of DNA with two distinct characteristics: the presence of nucleotide repeats and spacers. Repeated sequences of nucleotides are distributed throughout a CRISPR region with short segments of foreign DNA (spacers) interspersed among the repeated sequences. In the type II CRISPR/Cas system, spacers are integrated within the CRISPR genomic loci and transcribed and processed into short CRISPR RNA (crRNA). These crRNAs anneal to trans-activating crRNAs (tracrRNAs) and direct sequence-specific cleavage and silencing of pathogenic DNA by Cas proteins. Target recognition by the Cas9 protein requires a "seed" sequence within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA-binding region. The CRISPR/Cas system can thereby be retargeted to cleave virtually any DNA sequence by redesigning the crRNA. The crRNA and tracrRNA in the native system can be simplified into a single guide RNA (sgRNA) of approximately 100 nucleotides for use in genetic engineering. The CRISPR/Cas system is directly portable to human cells by co-delivery of plasmids expressing the Cas9 endo-nuclease and the necessary crRNA components. Different variants of Cas proteins may be used to reduce targeting limitations (e.g., orthologs of Cas9, such as Cpf1).

**[001169]** Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a CRISPR method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3.

**[001170]** Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a CRISPR method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL12, IL-15, and IL-21.

**[001171]** Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a CRISPR method, and which may be used in accordance with embodiments of the present invention, are described in U.S. Patent Nos. 8,697,359; 8,993,233; 8,795,965; 8,771,945; 8,889,356; 8,865,406; 8,999,641; 8,945,839; 8,932,814; 8,871,445; 8,906,616; and 8,895,308, which are incorporated by reference herein. Resources for carrying out CRISPR methods, such as plasmids for expressing CRISPR/Cas9 and CRISPR/Cpf1, are commercially available from companies such as GenScript.

**[001172]** In an embodiment, genetic modifications of populations of TILs, as described herein, may be performed using the CRISPR/Cpf1 system as described in U.S. Patent No. US 9790490, the disclosure of which is incorporated by reference herein.

**[001173]** A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a TALE method. According to particular embodiments, the use of a TALE method during the TIL expansion process causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a TALE method during the TIL expansion process causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

**[001174]** TALE stands for "Transcription Activator-Like Effector" proteins, which include TALENs ("Transcription Activator-Like Effector Nucleases"). A method of using a TALE system for gene editing may also be referred to herein as a TALE method. TALEs are

naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair. TALE specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues (RVDs). Modular TALE repeats are linked together to recognize contiguous DNA sequences. A specific RVD in the DNA-binding domain recognizes a base in the target locus, providing a structural feature to assemble predictable DNA-binding domains. The DNA binding domains of a TALE are fused to the catalytic domain of a type IIS FokI endonuclease to make a targetable TALE nuclease. To induce site-specific mutation, two individual TALEN arms, separated by a 14-20 base pair spacer region, bring FokI monomers in close proximity to dimerize and produce a targeted double-strand break.

**[001175]** Several large, systematic studies utilizing various assembly methods have indicated that TALE repeats can be combined to recognize virtually any user-defined sequence. Custom-designed TALE arrays are also commercially available through Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA). TALE and TALEN methods suitable for use in the present invention are described in U.S. Patent Application Publication Nos. US 2011/0201118 A1; US 2013/0117869 A1; US 2013/0315884 A1; US 2015/0203871 A1 and US 2016/0120906 A1, the disclosures of which are incorporated by reference herein.

**[001176]** Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a TALE method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3.

**[001177]** Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a TALE method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL12, IL-15, and IL-21.

**[001178]** Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a TALE method, and which may be used in accordance with

embodiments of the present invention, are described in U.S. Patent No. 8,586,526, which is incorporated by reference herein.

**[001179]** A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (e.g., process GEN 3) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a zinc finger or zinc finger nuclease method. According to particular embodiments, the use of a zinc finger method during the TIL expansion process causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a zinc finger method during the TIL expansion process causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

**[001180]** An individual zinc finger contains approximately 30 amino acids in a conserved ββα configuration. Several amino acids on the surface of the α-helix typically contact 3 bp in the major groove of DNA, with varying levels of selectivity. Zinc fingers have two protein domains. The first domain is the DNA binding domain, which includes eukaryotic transcription factors and contain the zinc finger. The second domain is the nuclease domain, which includes the FokI restriction enzyme and is responsible for the catalytic cleavage of DNA.

**[001181]** The DNA-binding domains of individual ZFNs typically contain between three and six individual zinc finger repeats and can each recognize between 9 and 18 base pairs. If the zinc finger domains are specific for their intended target site then even a pair of 3-finger ZFNs that recognize a total of 18 base pairs can, in theory, target a single locus in a mammalian genome. One method to generate new zinc-finger arrays is to combine smaller zinc-finger "modules" of known specificity. The most common modular assembly process involves combining three separate zinc fingers that can each recognize a 3 base pair DNA sequence to generate a 3-finger array that can recognize a 9 base pair target site. Alternatively, selection-based approaches, such as oligomerized pool engineering (OPEN) can be used to select for new zinc-finger arrays from randomized libraries that take into consideration context-dependent interactions between neighboring fingers. Engineered zinc fingers are available commercially; Sangamo Biosciences (Richmond, CA, USA) has developed a propriety platform (CompoZr®) for zinc-finger construction in partnership with Sigma–Aldrich (St. Louis, MO, USA).

**[001182]** Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a zinc finger method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3.

**[001183]** Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a zinc finger method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL12, IL-15, and IL-21.

**[001184]** Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a zinc finger method, which may be used in accordance with embodiments of the present invention, are described in U.S. Patent Nos. 6,534,261, 6,607,882, 6,746,838, 6,794,136, 6,824,978, 6,866,997, 6,933,113, 6,979,539, 7,013,219, 7,030,215, 7,220,719, 7,241,573, 7,241,574, 7,585,849, 7,595,376, 6,903,185, and 6,479,626, which are incorporated by reference herein.

**[001185]** Other examples of systems, methods, and compositions for altering the expression of a target gene sequence by a zinc finger method, which may be used in accordance with embodiments of the present invention, are described in Beane, *et al.*, *Mol. Therapy*, **2015,** 23 1380-1390, the disclosure of which is incorporated by reference herein.

**[001186]** In some embodiments, the TILs are optionally genetically engineered to include additional functionalities, including, but not limited to, a high-affinity T cell receptor (TCR), *e.g.*, a TCR targeted at a tumor-associated antigen such as MAGE-1, HER2, or NY-ESO-1, or a chimeric antigen receptor (CAR) which binds to a tumor-associated cell surface molecule (*e.g.*, mesothelin) or lineage-restricted cell surface molecule (*e.g.*, CD19). In certain embodiments, the method comprises genetically engineering a population of TILs to include a high-affinity T cell receptor (TCR), *e.g.*, a TCR targeted at a tumor-associated antigen such as MAGE-1, HER2, or NY-ESO-1, or a chimeric antigen receptor (CAR) which binds to a tumor-associated cell surface molecule (*e.g.*, mesothelin) or lineage-restricted cell surface molecule (*e.g.*, CD19). Aptly, the population of TILs may be a first population, a second population and/or a third population as described herein.

### K. Closed Systems for TIL Manufacturing

**[001187]** The present invention provides for the use of closed systems during the TIL culturing process. Such closed systems allow for preventing and/or reducing microbial contamination, allow for the use of fewer flasks, and allow for cost reductions. In some embodiments, the closed system uses two containers.

**[001188]** Such closed systems are well-known in the art and can be found, for example, at http://www.fda.gov/cber/guidelines.htm and https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076779.htm.

**[001189]** Sterile connecting devices (STCDs) produce sterile welds between two pieces of compatible tubing. This procedure permits sterile connection of a variety of containers and tube diameters. In some embodiments, the closed systems include luer lock and heat sealed systems as described in for example, Example G. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in Example G is employed. In some embodiments, the TILs are formulated into a final product formulation container according to the method described in Example G, section "Final Formulation and Fill".

**[001190]** In some embodiments, the closed system uses one container from the time the tumor fragments are obtained until the TILs are ready for administration to the patient or cryopreserving. In some embodiments when two containers are used, the first container is a closed G-container and the population of TILs is centrifuged and transferred to an infusion bag without opening the first closed G-container. In some embodiments, when two containers are used, the infusion bag is a HypoThermosol-containing infusion bag. A closed system or closed TIL cell culture system is characterized in that once the tumor sample and/or tumor fragments have been added, the system is tightly sealed from the outside to form a closed environment free from the invasion of bacteria, fungi, and/or any other microbial contamination.

**[001191]** In some embodiments, the reduction in microbial contamination is between about 5% and about 100%. In some embodiments, the reduction in microbial contamination is between about 5% and about 95%. In some embodiments, the reduction in microbial contamination is between about 5% and about 90%. In some embodiments, the reduction in

microbial contamination is between about 10% and about 90%. In some embodiments, the reduction in microbial contamination is between about 15% and about 85%. In some embodiments, the reduction in microbial contamination is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or about 100%.

**[001192]** The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

**[001193]** Moreover, pH, carbon dioxide partial pressure and oxygen partial pressure of the TIL cell culture environment each vary as the cells are cultured. Consequently, even though a medium appropriate for cell culture is circulated, the closed environment still needs to be constantly maintained as an optimal environment for TIL proliferation. To this end, it is desirable that the physical factors of pH, carbon dioxide partial pressure and oxygen partial pressure within the culture liquid of the closed environment be monitored by means of a sensor, the signal whereof is used to control a gas exchanger installed at the inlet of the culture environment, and the that gas partial pressure of the closed environment be adjusted in real time according to changes in the culture liquid so as to optimize the cell culture environment. In some embodiments, the present invention provides a closed cell culture system which incorporates at the inlet to the closed environment a gas exchanger equipped with a monitoring device which measures the pH, carbon dioxide partial pressure and oxygen partial pressure of the closed environment, and optimizes the cell culture environment by automatically adjusting gas concentrations based on signals from the monitoring device.

**[001194]** In some embodiments, the pressure within the closed environment is continuously or intermittently controlled. That is, the pressure in the closed environment can be varied by means of a pressure maintenance device for example, thus ensuring that the space is suitable for growth of TILs in a positive pressure state, or promoting exudation of fluid in a negative pressure state and thus promoting cell proliferation. By applying negative pressure intermittently, moreover, it is possible to uniformly and efficiently replace the circulating liquid in the closed environment by means of a temporary shrinkage in the volume of the closed environment.

**[001195]** In some embodiments, optimal culture components for proliferation of the TILs can be substituted or added, and including factors such as IL-2 and/or OKT3, as well as combination, can be added.

### L. Optional Cryopreservation of TILs

**[001196]** Either the bulk TIL population (for example the second population of TILs) or the expanded population of TILs (for example the third population of TILs) can be optionally cryopreserved. In some embodiments, cryopreservation occurs on the therapeutic TIL population. In some embodiments, cryopreservation occurs on the TILs harvested after the second expansion. In some embodiments, cryopreservation occurs on the TILs in exemplary Step F of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, the TILs are cryopreserved in the infusion bag. In some embodiments, the TILs are cryopreserved prior to placement in an infusion bag. In some embodiments, the TILs are cryopreserved and not placed in an infusion bag. In some embodiments, cryopreservation is performed using a cryopreservation medium. In some embodiments, the cryopreservation media contains dimethylsulfoxide (DMSO). This is generally accomplished by putting the TIL population into a freezing solution, *e.g.* 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See, Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986.

**[001197]** When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

**[001198]** In a preferred embodiment, a population of TILs is cryopreserved using CS10 cryopreservation media (CryoStor 10, BioLife Solutions). In a preferred embodiment, a population of TILs is cryopreserved using a cryopreservation media containing dimethylsulfoxide (DMSO). In a preferred embodiment, a population of TILs is cryopreserved using a 1:1 (vol:vol) ratio of CS10 and cell culture media. In a preferred embodiment, a population of TILs is cryopreserved using about a 1:1 (vol:vol) ratio of CS10 and cell culture media, further comprising additional IL-2.

**[001199]** As discussed above, and exemplified in Steps A through E as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), cryopreservation can occur at numerous points throughout the TIL expansion process. In some embodiments, the expanded population of TILs after the second expansion (as provided for example, according to Step D of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) can be cryopreserved. Cryopreservation can be generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986. In some embodiments, the TILs are cryopreserved in 5% DMSO. In some embodiments, the TILs are cryopreserved in cell culture media plus 5% DMSO. In some embodiments, the TILs are cryopreserved according to the methods provided in Example D.

**[001200]** When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

**[001201]** In some cases, the Step B TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to Step C and Step D and then cryopreserved after Step D. Similarly, in the case where genetically modified TILs will be used in therapy, the Step B or Step D TIL populations can be subjected to genetic modifications for suitable treatments.

### M. Phenotypic Characteristics of Expanded TILs

**[001202]** In some embodiment, the TILs are analyzed for expression of numerous phenotype markers after expansion, including those described herein and in the Examples. In an embodiment, expression of one or more phenotypic markers is examined. In some embodiments, the phenotypic characteristics of the TILs are analyzed after the first expansion in Step B. In some embodiments, the phenotypic characteristics of the TILs are analyzed during the transition in Step C. In some embodiments, the phenotypic characteristics of the TILs are analyzed during the transition according to Step C and after cryopreservation. In some embodiments, the phenotypic characteristics of the TILs are analyzed after the second

expansion according to Step D. In some embodiments, the phenotypic characteristics of the TILs are analyzed after two or more expansions according to Step D.

**[001203]** In some embodiments, the marker is selected from the group consisting of CD8 and CD28. In some embodiments, expression of CD8 is examined. In some embodiments, expression of CD28 is examined. In some embodiments, the expression of CD8 and/or CD28 is higher on TILs produced according the current invention process, as compared to other processes (*e.g.*, the Gen 3 process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as compared to the 2A process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, the expression of CD8 is higher on TILs produced according the current invention process, as compared to other processes (*e.g.*, the Gen 3 process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8B), as compared to the 2A process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, the expression of CD28 is higher on TILs produced according the current invention process, as compared to other processes (*e.g.*, the Gen 3 process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as compared to the 2A process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8A)). In some embodiments, high CD28 expression is indicative of a younger, more presisitent TIL phenotype. In an embodiment, expression of one or more regulatory markers is measured.

**[001204]** In an embodiment, no selection of the first population of TILs, second population of TILs, third population of TILs, or harvested TIL population based on CD8 and/or CD28 expression is performed during any of the steps for the method for expanding tumor infiltrating lymphocytes (TILs) described herein.

**[001205]** In some embodiments, the percentage of central memory cells is higher on TILs produced according the current invention process, as compared to other processes (*e.g.*, the Gen 3 process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as compared to the 2A process as provided for example in Figure 8 (in particular, *e.g.*, Figure 8A)). In some embodiments the memory marker for central memory cells is selected from the group consisting of CCR7 and CD62L.

**[001206]** In some embodiments, the CD4+ and/or CD8+ TIL Memory subsets can be divided into different memory subsets. In some embodiments, the CD4+ and/or CD8+ TILs comprise the naïve (CD45RA+CD62L+) TILS. In some embodiments, the CD4+ and/or CD8+ TILs

comprise the central memory (CM; CD45RA-CD62L+) TILs. In some embodiments, the CD4+ and/or CD8+ TILs comprise the effector memory (EM; CD45RA-CD62L-) TILs. In some embodiments, the CD4+ and/or CD8+ TILs comprise the, RA+ effector memory/effector (TEMRA/TEFF; CD45RA+CD62L+) TILs.

**[001207]** In some embodiments, the TILs express one more markers selected from the group consisting of granzyme B, perforin, and granulysin. In some embodiments, the TILs express granzyme B. In some embodiments, the TILs express perforin. In some embodiments, the TILs express granulysin.

**[001208]** In an embodiment, restimulated TILs can also be evaluated for cytokine release, using cytokine release assays. In some embodiments, TILs can be evaluated for interferon-γ (IFN-γ) secretion. In some embodiments, the IFN-γ secretion is measured by an ELISA assay. In some embodiments, the IFN-γ secretion is measured by an ELISA assay after the rapid second expansion step, after Step D as provided in for example, Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, TIL health is measured by IFN-gamma (IFN-γ) secretion. In some embodiments, IFN-γ secretion is indicative of active TILs. In some embodiments, a potency assay for IFN-γ production is employed. IFN-γ production is another measure of cytotoxic potential. IFN-γ production can be measured by determining the levels of the cytokine IFN-γ in the media of TIL stimulated with antibodies to CD3, CD28, and CD137/4-1BB. IFN-γ levels in media from these stimulated TIL can be determined using by measuring IFN-γ release. In some embodiments, an increase in IFN-γ production in for example Step D in the Gen 3 process as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) TILs as compared to for example Step D in the 2A process as provided in Figure 8 (in particular, *e.g.*, Figure 8A) is indicative of an increase in cytotoxic potential of the Step D TILs. In some embodiments, IFN-γ secretion is increased one-fold, two-fold, three-fold, four-fold, or five-fold or more. In some embodiments, IFN-γ secretion is increased one-fold. In some embodiments, IFN-γ secretion is increased two-fold. In some embodiments, IFN-γ secretion is increased three-fold. In some embodiments, IFN-γ secretion is increased four-fold. In some embodiments, IFN-γ secretion is increased five-fold. In some embodiments, IFN-γ is measured using a Quantikine ELISA kit. In some embodiments, IFN-γ is measured in TILs *ex vivo*. In some embodiments, IFN-γ is measured in TILs *ex vivo*, including TILs produced by the methods of the present invention, including, for example Figure 8B methods.

**[001209]** In some embodiments, TILs capable of at least one-fold, two-fold, three-fold, four-fold, or five-fold or more IFN-γ secretion are TILs produced by the expansion methods of the present invention, including, for example Figure 8B and/or Figure 8C methods. In some embodiments, TILs capable of at least one-fold more IFN-γ secretion are TILs produced by the expansion methods of the present invention, including, for example Figure 8B and/or Figure 8C methods. In some embodiments, TILs capable of at least two-fold more IFN-γ secretion are TILs produced by the expansion methods of the present invention, including, for example Figure 8B and/or Figure 8C methods. In some embodiments, TILs capable of at least three-fold more IFN-γ secretion are TILs produced by the expansion methods of the present invention, including, for example Figure 8B and/or Figure 8C methods. In some embodiments, TILs capable of at least four-fold more IFN-γ secretion are TILs produced by the expansion methods of the present invention, including, for example Figure 8B and/or Figure 8C methods. In some embodiments, TILs capable of at least five-fold more IFN-γ secretion are TILs produced by the expansion methods of the present invention, including, for example Figure 8B and/or Figure 8C methods

**[001210]** The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using other methods than those provide herein including, for example, methods other than those embodied in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using methods referred to as process 2A, as exemplified in Figure 8 (in particular, *e.g.*, Figure 8A). In some embodiments, the TILs obtained in the first expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the

immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCRα/β). In some embodiments, the process as described herein (*e.g.*, the Gen 3 process) shows higher clonal diversity as compared to other processes, for example the process referred to as the Gen 2 based on the number of unique peptide CDRs within the sample (*see*, for example Figures 12-14).

**[001211]** In some embodiments, the activation and exhaustion of TILs can be determined by examining one or more markers. In some embodiments, the activation and exhaustion can be determined using multicolor flow cytometry. In some embodiments, the activation and exhaustion of markers include but not limited to one or more markers selected from the group consisting of CD3, PD-1, 2B4/CD244, CD8, CD25, BTLA, KLRG, TIM-3, CD194/CCR4, CD4, TIGIT, CD183, CD69, CD95, CD127, CD103, and/or LAG-3). In some embodiments, the activation and exhaustion of markers include but not limited to one or more markers selected from the group consisting of BTLA, CTLA-4, ICOS, Ki67, LAG-3, PD-1, TIGIT, and/or TIM-3. In some embodiments, the activation and exhaustion of markers include but not limited to one or more markers selected from the group consisting of BTLA, CTLA-4, ICOS, Ki67, LAG-3, CD103+/CD69+, CD103+/CD69-, PD-1, TIGIT, and/or TIM-3. In some embodiments, the T-cell markers (including activation and exhaustion markers) can be determined and/or analyzed to examine T-cell activation, inhibition, or function. In some embodiments, the T-cell markers can include but are not limited to one or more markers selected from the group consisting of TIGIT, CD3, FoxP3, Tim-3, PD-1, CD103, CTLA-4, LAG-3, BTLA-4, ICOS, Ki67, CD8, CD25, CD45, CD4, and/or CD59.

**[001212]** In some embodiments, the phenotypic characterization is examined after cryopreservation.

### M. Additional Process Embodiments

**[001213]** In some embodiments, the invention provides a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising: (a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject into multiple tumor fragments; (b) performing a

priming first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3, wherein the priming first expansion is performed for about 1 to 7 days or about about 1 to 8 days to obtain the second population of TILs, wherein the second population of TILs is greater in number than the first population of TILs; (c) performing a rapid second expansion by contacting the second population of TILs with a cell culture medium comprising IL-2, OKT-3 and exogenous antigen presenting cells (APCs) to produce a third population of TILs, wherein the rapid second expansion is performed for about 1 to 11 days or about 1 to 10 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs; and (d) harvesting the therapeutic population of TILs obtained from step (c). In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, or about 2 to 4 days, and then (2) effecting the transfer of the second population of TILs from the small scale culture to a second container larger than the first container, e.g., a G-REX 500MCS container, wherein in the second container the second population of TILs from the small scale culture is cultured in a larger scale culture for a period of about 4 to 7 days,or about 4 to 8 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a first small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the second population of TILs from the first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 4 to 7 days, or about about 4 to 8 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, or about 2 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container, e.g., G-REX 500MCS containers,

wherein in each second container the portion of the second population of TILs transferred from the small scale culture to such second container is cultured in a larger scale culture for a period of about 4 to 7 days, or about 4 to 8 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, e.g., G-REX 500MCS containers, wherein in each second container the portion of the second population of TILs transferred from the small scale culture to such second container is cultured in a larger scale culture for a period of about 5 to 7 days.

**[001214]** In some embodiments, the invention provides a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising: (a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject into multiple tumor fragments; (b) performing a priming first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3, wherein the priming first expansion is performed for about 1 to 8 days to obtain the second population of TILs, wherein the second population of TILs is greater in number than the first population of TILs; (c) performing a rapid second expansion by contacting the second population of TILs with a cell culture medium comprising IL-2, OKT-3 and exogenous antigen presenting cells (APCs) to produce a third population of TILs, wherein the rapid second expansion is performed for about 1 to 8 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs; and (d) harvesting the therapeutic population of TILs obtained from step (c). In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 2 to 4 days, and then (2) effecting the transfer of the second population of TILs from the small scale culture to a second container larger than the first container, *e.g.*, a G-REX 500MCS container, wherein in the second container the second population of TILs from the small scale culture is cultured in a larger scale culture for a period of about 4 to 8 days. In some embodiments, the step of rapid expansion is split into a

plurality of steps to achieve a scaling out of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a first small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 2 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the second population of TILs from the first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 4 to 6 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 2 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the second population of TILs transferred from the small scale culture to such second container is cultured in a larger scale culture for a period of about 4 to 6 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the second population of TILs transferred from the small scale culture to such second container is cultured in a larger scale culture for a period of about 4 to 5 days.

**[001215]** In some embodiments, the invention provides a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising: (a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject into multiple tumor fragments; (b) performing a priming first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3, wherein the priming first expansion is performed for about 1 to

7 days to obtain the second population of TILs, wherein the second population of TILs is greater in number than the first population of TILs; (c) performing a rapid second expansion by contacting the second population of TILs with a cell culture medium comprising IL-2, OKT-3 and exogenous antigen presenting cells (APCs) to produce a third population of TILs, wherein the rapid second expansion is performed for about 1 to 11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs; and (d) harvesting the therapeutic population of TILs obtained from step (c). In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (2) effecting the transfer of the second population of TILs from the small scale culture to a second container larger than the first container, *e.g.*, a G-REX 500MCS container, wherein in the second container the second population of TILs from the small scale culture is cultured in a larger scale culture for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a first small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the second population of TILs from the first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the second population of TILs transferred from the small scale culture to such second container is cultured in a larger scale culture for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the

culture by: (1) performing the rapid second expansion by culturing the second population of TILs in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 4 days, and then (2) effecting the transfer and apportioning of the second population of TILs from the first small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the second population of TILs transferred from the small scale culture to such second container is cultured in a larger scale culture for a period of about 5 days.

**[001216]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by contacting the first population of TILs with a culture medium which further comprises exogenous antigen-presenting cells (APCs), wherein the number of APCs in the culture medium in step (c) is greater than the number of APCs in the culture medium in step (b).

**[001217]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the culture medium is supplemented with additional exogenous APCs.

**[001218]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 20:1.

**[001219]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 10:1.

**[001220]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 9:1.

**[001221]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs

added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 8:1.

**[001222]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 7:1.

**[001223]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 6:1.

**[001224]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 5:1.

**[001225]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 4:1.

**[001226]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 3:1.

**[001227]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.9:1.

**[001228]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.8:1.

**[001229]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.7:1.

**[001230]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.6:1.

**[001231]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.5:1.

**[001232]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.4:1.

**[001233]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.3:1.

**[001234]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.2:1.

**[001235]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2.1:1.

**[001236]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 1.1:1 to at or about 2:1.

**[001237]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 10:1.

**[001238]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 5:1.

**[001239]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 4:1.

**[001240]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 3:1.

**[001241]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.9:1.

**[001242]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.8:1.

**[001243]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.7:1.

**[001244]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.6:1.

**[001245]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.5:1.

**[001246]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.4:1.

**[001247]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.3:1.

**[001248]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.2:1.

**[001249]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is selected from a range of from at or about 2:1 to at or about 2.1:1.

**[001250]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is at or about 2:1.

**[001251]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of number of APCs added in the rapid second expansion to the number of APCs added in step (b) is at or about 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, 2:1, 2.1:1, 2.2:1, 2.3:1, 2.4:1, 2.5:1, 2.6:1, 2.7:1, 2.8:1, 2.9:1, 3:1, 3.1:1, 3.2:1, 3.3:1, 3.4:1, 3.5:1, 3.6:1, 3.7:1, 3.8:1, 3.9:1, 4:1, 4.1:1, 4.2:1, 4.3:1, 4.4:1, 4.5:1, 4.6:1, 4.7:1, 4.8:1, 4.9:1, or 5:1.

**[001252]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the number of APCs added in

the primary first expansion is at or about $1\times10^8$, $1.1\times10^8$, $1.2\times10^8$, $1.3\times10^8$, $1.4\times10^8$, $1.5\times10^8$, $1.6\times10^8$, $1.7\times10^8$, $1.8\times10^8$, $1.9\times10^8$, $2\times10^8$, $2.1\times10^8$, $2.2\times10^8$, $2.3\times10^8$, $2.4\times10^8$, $2.5\times10^8$, $2.6\times10^8$, $2.7\times10^8$, $2.8\times10^8$, $2.9\times10^8$, $3\times10^8$, $3.1\times10^8$, $3.2\times10^8$, $3.3\times10^8$, $3.4\times10^8$ or $3.5\times10^8$ APCs, and such that the number of APCs added in the rapid second expansion is at or about $3.5\times10^8$, $3.6\times10^8$, $3.7\times10^8$, $3.8\times10^8$, $3.9\times10^8$, $4\times10^8$, $4.1\times10^8$, $4.2\times10^8$, $4.3\times10^8$, $4.4\times10^8$, $4.5\times10^8$, $4.6\times10^8$, $4.7\times10^8$, $4.8\times10^8$, $4.9\times10^8$, $5\times10^8$, $5.1\times10^8$, $5.2\times10^8$, $5.3\times10^8$, $5.4\times10^8$, $5.5\times10^8$, $5.6\times10^8$, $5.7\times10^8$, $5.8\times10^8$, $5.9\times10^8$, $6\times10^8$, $6.1\times10^8$, $6.2\times10^8$, $6.3\times10^8$, $6.4\times10^8$, $6.5\times10^8$, $6.6\times10^8$, $6.7\times10^8$, $6.8\times10^8$, $6.9\times10^8$, $7\times10^8$, $7.1\times10^8$, $7.2\times10^8$, $7.3\times10^8$, $7.4\times10^8$, $7.5\times10^8$, $7.6\times10^8$, $7.7\times10^8$, $7.8\times10^8$, $7.9\times10^8$, $8\times10^8$, $8.1\times10^8$, $8.2\times10^8$, $8.3\times10^8$, $8.4\times10^8$, $8.5\times10^8$, $8.6\times10^8$, $8.7\times10^8$, $8.8\times10^8$, $8.9\times10^8$, $9\times10^8$, $9.1\times10^8$, $9.2\times10^8$, $9.3\times10^8$, $9.4\times10^8$, $9.5\times10^8$, $9.6\times10^8$, $9.7\times10^8$, $9.8\times10^8$, $9.9\times10^8$ or $1\times10^9$ APCs.

**[001253]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the number of APCs added in the primary first expansion is selected from the range of at or about $1\times10^8$ APCs to at or about $3.5\times10^8$ APCs, and wherein the number of APCs added in the rapid second expansion is selected from the range of at or about $3.5\times10^8$ APCs to at or about $1\times10^9$ APCs.

**[001254]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the number of APCs added in the primary first expansion is selected from the range of at or about $1.5\times10^8$ APCs to at or about $3\times10^8$ APCs, and wherein the number of APCs added in the rapid second expansion is selected from the range of at or about $4\times10^8$ APCs to at or about $7.5\times10^8$ APCs.

**[001255]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the number of APCs added in the primary first expansion is selected from the range of at or about $2\times10^8$ APCs to at or about $2.5\times10^8$ APCs, and wherein the number of APCs added in the rapid second expansion is selected from the range of at or about $4.5\times10^8$ APCs to at or about $5.5\times10^8$ APCs.

**[001256]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that at or about $2.5\times10^8$ APCs are added to the primary first expansion and at or about $5\times10^8$ APCs are added to the rapid second expansion.

**[001257]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the antigen-presenting cells are peripheral blood mononuclear cells (PBMCs).

**[001258]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple tumor fragments are distributed into a plurality of separate containers, in each of which separate containers the first population of TILs is obtained in step (a), the second population of TILs is obtained in step (b), and the third population of TILs is obtained in step (c), and the therapeutic populations of TILs from the plurality of containers in step (c) are combined to yield the harvested TIL population from step (d).

**[001259]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple tumors are evenly distributed into the plurality of separate containers.

**[001260]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the plurality of separate containers comprises at least two separate containers.

**[001261]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the plurality of separate containers comprises from two to twenty separate containers.

**[001262]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the plurality of separate containers comprises from two to fifteen separate containers.

**[001263]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the plurality of separate containers comprises from two to ten separate containers.

**[001264]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the plurality of separate containers comprises from two to five separate containers.

**[001265]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the plurality of separate

containers comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 separate containers.

**[001266]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that for each container in which the priming first expansion is performed on a first population of TILs in step (b) the rapid second expansion in step (c) is performed in the same container on the second population of TILs produced from such first population of TILs.

**[001267]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each of the separate containers comprises a first gas-permeable surface area.

**[001268]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple tumor fragments are distributed in a single container.

**[001269]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the single container comprises a first gas-permeable surface area.

**[001270]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about one cell layer to at or about three cell layers.

**[001271]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 1.5 cell layers to at or about 2.5 cell layers.

**[001272]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 2 cell layers.

**[001273]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3 cell layers.

**[001274]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 3 cell layers to at or about 10 cell layers.

**[001275]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 4 cell layers to at or about 8 cell layers.

**[001276]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 3, 4, 5, 6, 7, 8, 9 or 10 cell layers.

**[001277]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8 cell layers.

**[001278]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the priming first expansion is performed in a first container comprising a first gas-permeable surface area and in step (c) the rapid second expansion is performed in a second container comprising a second gas-permeable surface area.

**[001279]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the second container is larger than the first container.

**[001280]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of

TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about one cell layer to at or about three cell layers.

**[001281]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 1.5 cell layers to at or about 2.5 cell layers.

**[001282]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 2 cell layers.

**[001283]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3 cell layers.

**[001284]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the second gas-permeable surface area at an average thickness of at or about 3 cell layers to at or about 10 cell layers.

**[001285]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the second gas-permeable surface area at an average thickness of at or about 4 cell layers to at or about 8 cell layers.

**[001286]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the second gas-permeable surface area at an average thickness of at or about 3, 4, 5, 6, 7, 8, 9 or 10 cell layers.

**[001287]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable modified such that in step (c) the APCs are layered onto the second gas-permeable surface area at an average thickness of at or about 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8 cell layers.

**[001288]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the priming first expansion is performed in a first container comprising a first gas-permeable surface area and in step (c) the rapid second expansion is performed in the first container.

**[001289]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about one cell layer to at or about three cell layers.

**[001290]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 1.5 cell layers to at or about 2.5 cell layers.

**[001291]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 2 cell layers.

**[001292]** In another embodiment, the invention provides the method described any of the preceding paragraphs as applicable above modified such that in step (b) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3 cell layers.

**[001293]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 3 cell layers to at or about 10 cell layers.

**[001294]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 4 cell layers to at or about 8 cell layers.

**[001295]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered

onto the first gas-permeable surface area at an average thickness of at or about 3, 4, 5, 6, 7, 8, 9 or 10 cell layers.

**[001296]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (c) the APCs are layered onto the first gas-permeable surface area at an average thickness of at or about 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8 cell layers.

**[001297]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:10.

**[001298]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:9.

**[001299]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:8.

**[001300]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in

step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:7.

**[001301]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:6.

**[001302]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:5.

**[001303]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:4.

**[001304]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:3.

**[001305]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.1 to at or about 1:2.

**[001306]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.2 to at or about 1:8.

**[001307]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.3 to at or about 1:7.

**[001308]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.4 to at or about 1:6.

**[001309]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in

step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.5 to at or about 1:5.

**[001310]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.6 to at or about 1:4.

**[001311]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.7 to at or about 1:3.5.

**[001312]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.8 to at or about 1:3.

**[001313]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:1.9 to at or about 1:2.5.

**[001314]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from the range of at or about 1:2.

**[001315]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the primary first expansion is performed by supplementing the cell culture medium of the first population of TILs with additional antigen-presenting cells (APCs), wherein the number of APCs added in step (c) is greater than the number of APCs added in step (b), and wherein the ratio of the average number of layers of APCs layered in step (b) to the average number of layers of APCs layered in step (c) is selected from at or about 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9, 1:2, 1:2.1, 1:2.2, 1:2.3, 1:2.4, 1:2.5, 1:2.6, 1:2.7, 1:2.8, 1:2.9, 1:3, 1:3.1, 1:3.2, 1:3.3, 1:3.4, 1:3.5, 1:3.6, 1:3.7, 1:3.8, 1:3.9, 1:4, 1:4.1, 1:4.2, 1:4.3, 1:4.4, 1:4.5, 1:4.6, 1:4.7, 1:4.8, 1:4.9, 1:5, 1:5.1, 1:5.2, 1:5.3, 1:5.4, 1:5.5, 1:5.6, 1:5.7, 1:5.8, 1:5.9, 1:6, 1:6.1, 1:6.2, 1:6.3, 1:6.4, 1:6.5, 1:6.6, 1:6.7, 1:6.8, 1:6.9, 1:7, 1:7.1, 1:7.2, 1:7.3, 1:7.4, 1:7.5, 1:7.6, 1:7.7, 1:7.8, 1:7.9, 1:8, 1:8.1, 1:8.2, 1:8.3, 1:8.4, 1:8.5, 1:8.6, 1:8.7, 1:8.8, 1:8.9, 1:9, 1:9.1, 1:9.2, 1:9.3, 1:9.4, 1:9.5, 1:9.6, 1:9.7, 1:9.8, 1:9.9 or 1:10.

**[001316]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is at or about 1.5:1 to at or about 100:1.

**[001317]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is at or about 50:1.

**[001318]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is at or about 25:1.

**[001319]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is at or about 20:1.

**[001320]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is at or about 10:1.

**[001321]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the second population of TILs is at least at or about 50-fold greater in number than the first population of TILs.

**[001322]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the second population of TILs is at least at or about 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14-, 15-, 16-, 17-, 18-, 19-, 20-, 21-, 22-, 23-, 24-, 25-, 26-, 27-, 28-, 29-, 30-, 31-, 32-, 33-, 34-, 35-, 36-, 37-, 38-, 39-, 40-, 41-, 42-, 43-, 44-, 45-, 46-, 47-, 48-, 49- or 50-fold greater in number than the first population of TILs.

**[001323]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that at or about 2 days or at or about 3 days after the commencement of the second period in step (c), the cell culture medium is supplemented with additional IL-2.

**[001324]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified to further comprise the step of cryopreserving the harvested TIL population in step (d) using a cryopreservation process.

**[001325]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified to comprise performing after step (d) the additional step of (e) transferring the harvested TIL population from step (d) to an infusion bag that optionally contains HypoThermosol.

**[001326]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified to comprise the step of cryopreserving the infusion bag comprising the harvested TIL population in step (e) using a cryopreservation process.

**[001327]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the cryopreservation process is performed using a 1:1 ratio of harvested TIL population to cryopreservation media.

**[001328]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the antigen-presenting cells are peripheral blood mononuclear cells (PBMCs).

**[001329]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the PBMCs are irradiated and allogeneic.

**[001330]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the total number of APCs added to the cell culture in step (b) is $2.5 \times 10^8$.

**[001331]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the total number of APCs added to the cell culture in step (c) is $5 \times 10^8$.

**[001332]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the APCs are PBMCs.

**[001333]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the PBMCs are irradiated and allogeneic.

**[001334]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the antigen-presenting cells are artificial antigen-presenting cells.

**[001335]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the harvesting in step (d) is performed using a membrane-based cell processing system.

**[001336]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the harvesting in step (d) is performed using a LOVO cell processing system.

**[001337]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 5 to at or about 60 fragments per container in step (b).

**[001338]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 10 to at or about 60 fragments per container in step (b).

**[001339]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 15 to at or about 60 fragments per container in step (b).

**[001340]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 20 to at or about 60 fragments per container in step (b).

**[001341]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 25 to at or about 60 fragments per container in step (b).

**[001342]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 30 to at or about 60 fragments per container in step (b).

**[001343]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 35 to at or about 60 fragments per container in step (b).

**[001344]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 40 to at or about 60 fragments per container in step (b).

**[001345]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 45 to at or about 60 fragments per container in step (b).

**[001346]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 50 to at or about 60 fragments per container in step (b).

**[001347]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or 60 fragment(s) per container in step (b).

**[001348]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 27 $mm^3$.

**[001349]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 20 $mm^3$ to at or about 50 $mm^3$.

**[001350]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 21 $mm^3$ to at or about 30 $mm^3$.

**[001351]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 22 $mm^3$ to at or about 29.5 $mm^3$.

**[001352]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 23 $mm^3$ to at or about 29 $mm^3$.

**[001353]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 24 $mm^3$ to at or about 28.5 $mm^3$.

**[001354]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 25 $mm^3$ to at or about 28 $mm^3$.

**[001355]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of at or about 26.5 $mm^3$ to at or about 27.5 $mm^3$.

**[001356]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each fragment has a volume of

at or about 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 $mm^3$.

**[001357]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 30 to at or about 60 fragments with a total volume of at or about 1300 $mm^3$ to at or about 1500 $mm^3$.

**[001358]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 50 fragments with a total volume of at or about 1350 $mm^3$.

**[001359]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the multiple fragments comprise at or about 50 fragments with a total mass of at or about 1 gram to at or about 1.5 grams.

**[001360]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the cell culture medium is provided in a container that is a G-container or a Xuri cellbag.

**[001361]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the IL-2 concentration in the cell culture medium is about 10,000 IU/mL to about 5,000 IU/mL.

**[001362]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the IL-2 concentration in the cell culture medium is about 6,000 IU/mL.

**[001363]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the cryopreservation media comprises dimethlysulfoxide (DMSO).

**[001364]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the cryopreservation media comprises 7% to 10% DMSO.

**[001365]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first period in step (b) is performed within a period of at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days.

**[001366]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the second period in step (c) is performed within a period of at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

**[001367]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first period in step (b) and the second period in step (c) are each individually performed within a period of at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days.

**[001368]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first period in step (b) and the second period in step (c) are each individually performed within a period of at or about 5 days, 6 days, or 7 days.

**[001369]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first period in step (b) and the second period in step (c) are each individually performed within a period of at or about 7 days.

**[001370]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 14 days to at or about 18 days.

**[001371]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 15 days to at or about 18 days.

**[001372]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 16 days to at or about 18 days.

**[001373]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 17 days to at or about 18 days.

**[001374]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 14 days to at or about 17 days.

**[001375]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 15 days to at or about 17 days.

**[001376]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 16 days to at or about 17 days.

**[001377]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 14 days to at or about 16 days.

**[001378]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 15 days to at or about 16 days.

**[001379]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 14 days.

**[001380]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 15 days.

**[001381]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 16 days.

**[001382]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 17 days.

**[001383]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 18 days.

**[001384]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 14 days or less.

**[001385]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 15 days or less.

**[001386]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 16 days or less.

**[001387]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that steps (a) through (d) are performed in a total of at or about 18 days or less.

**[001388]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs harvested in step (d) comprises sufficient TILs for a therapeutically effective dosage of the TILs.

**[001389]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the number of TILs sufficient for a therapeutically effective dosage is from at or about $2.3\times10^{10}$ to at or about $13.7\times10^{10}$.

**[001390]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the third population of TILs in step (c) provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality.

**[001391]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the third population of TILs in step (c) provides for at least a one-fold to five-fold or more interferon-gamma production as compared to TILs prepared by a process longer than 16 days.

**[001392]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the third population of TILs in step (c) provides for at least a one-fold to five-fold or more interferon-gamma production as compared to TILs prepared by a process longer than 17 days.

**[001393]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the third population of TILs

in step (c) provides for at least a one-fold to five-fold or more interferon-gamma production as compared to TILs prepared by a process longer than 18 days.

**[001394]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the effector T cells and/or central memory T cells obtained from the third population of TILs step (c) exhibit increased CD8 and CD28 expression relative to effector T cells and/or central memory T cells obtained from the second population of cells step (b).

**[001395]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a closed container.

**[001396]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a G-container.

**[001397]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-10.

**[001398]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-100.

**[001399]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-500.

**[001400]** In another embodiment, the invention provides the therapeutic population of tumor infiltrating lymphocytes (TILs) made by the method described in any of the preceding paragraphs as applicable above.

**[001401]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process in which the first expansion of TILs is performed without any added antigen-presenting cells (APCs) or OKT3.

**[001402]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process in which the first expansion of TILs is performed without any added antigen-presenting cells (APCs).

**[001403]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process in which the first expansion of TILs is performed without any added OKT3.

**[001404]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process in which the first expansion of TILs is performed with no added antigen-presenting cells (APCs) and no added OKT3.

**[001405]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process by a process longer than 16 days.

**[001406]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process by a process longer than 17 days.

**[001407]** In another embodiment, the invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) prepared from tumor tissue of a patient, wherein the therapeutic population of TILs provides for increased efficacy, increased interferon-gamma production, and/or increased polyclonality compared to TILs prepared by a process by a process longer than 18 days.

**[001408]** In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above that provides for increased interferon-gamma production.

**[001409]** In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above that provides for increased polyclonality.

**[001410]** In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above that provides for increased efficacy.

**[001411]** In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least one-fold more interferon-gamma production as compared to TILs prepared by a process longer than 16 days. In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least one-fold more interferon-gamma production as compared to TILs prepared by a process longer than 17 days. In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least one-fold more interferon-gamma production as compared to TILs prepared by a process longer than 18 days. In some embodiments, the TILs are rendered capable of the at least one-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001412]** In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least two-fold more interferon-gamma production as compared to TILs prepared by a process longer than 16 days. In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least two-fold more interferon-gamma production as compared to TILs prepared by a process longer than 17 days. In another embodiment, the invention

provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least two-fold more interferon-gamma production as compared to TILs prepared by a process longer than 18 days. In some embodiments, the TILs are rendered capable of the at least two-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001413]** In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least three-fold more interferon-gamma production as compared to TILs prepared by a process longer than 16 days. In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least three-fold more interferon-gamma production as compared to TILs prepared by a process longer than 17 days. In another embodiment, the invention provides for the therapeutic population of TILs described in any of the preceding paragraphs as applicable above modified such that the therapeutic population of TILs is capable of at least three-fold more interferon-gamma production as compared to TILs prepared by a process longer than 18 days. In some embodiments, the TILs are rendered capable of the at least three-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001414]** In another embodiment, the invention provides for a therapeutic population of tumor infiltrating lymphocytes (TILs) that is capable of at least one-fold more interferon-gamma production as compared to TILs prepared by a process in which the first expansion of TILs is performed without any added antigen-presenting cells (APCs). In some embodiments, the TILs are rendered capable of the at least one-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001415]** In another embodiment, the invention provides for a therapeutic population of tumor infiltrating lymphocytes (TILs) that is capable of at least one-fold more interferon-gamma

production as compared to TILs prepared by a process in which the first expansion of TILs is performed without any added OKT3. In some embodiments, the TILs are rendered capable of the at least one-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001416]** In another embodiment, the invention provides for a therapeutic population of TILs that is capable of at least two-fold more interferon-gamma production as compared to TILs prepared by a process in which the first expansion of TILs is performed without any added APCs. In some embodiments, the TILs are rendered capable of the at least two-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001417]** In another embodiment, the invention provides for a therapeutic population of TILs that is capable of at least two-fold more interferon-gamma production as compared to TILs prepared by a process in which the first expansion of TILs is performed without any added OKT3. In some embodiments, the TILs are rendered capable of the at least two-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001418]** In another embodiment, the invention provides for a therapeutic population of TILs that is capable of at least three-fold more interferon-gamma production as compared to TILs prepared by a process in which the first expansion of TILs is performed without any added APCs. In some embodiments, the TILs are rendered capable of the at least one-fold more interferon-gamma production due to the expansion process described herein, for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001419]** In another embodiment, the invention provides for a therapeutic population of TILs that is capable of at least three-fold more interferon-gamma production as compared to TILs prepared by a process in which the first expansion of TILs is performed without any added OKT3. In some embodiments, the TILs are rendered capable of the at least three-fold more interferon-gamma production due to the expansion process described herein, for example as

described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C).

**[001420]** In another embodiment, the invention provides a method of expanding T cells comprising: (a) performing a priming first expansion of a first population of T cells obtained from a donor by culturing the first population of T cells to effect growth and to prime an activation of the first population of T cells; (b) after the activation of the first population of T cells primed in step (a) begins to decay, performing a rapid second expansion of the first population of T cells by culturing the first population of T cells to effect growth and to boost the activation of the first population of T cells to obtain a second population of T cells; and (c) harvesting the second population of T cells. In another embodiment, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer of the first population of T cells from the small scale culture to a second container larger than the first container, e.g., a G-REX 500MCS container, and culturing the first population of T cells from the small scale culture in a larger scale culture in the second container for a period of about 4 to 7 days. In another embodiment, the step of rapid expansion is split into a plurality of steps to achieve a scaling out of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a first small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the first population of T cells from first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 4 to 7 days. In another embodiment, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container, e.g., G-REX 500MCS containers,

wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 4 to 7 days. In another embodiment, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, e.g., a G-REX 100MCS container, for a period of about 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, e.g., G-REX 500MCS containers, wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 5 days.

**[001421]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 2 to 4 days, and then (b) effecting the transfer of the first population of T cells from the small scale culture to a second container larger than the first container, *e.g.*, a G-REX 500MCS container, and culturing the first population of T cells from the small scale culture in a larger scale culture in the second container for a period of about 5 to 7 days.

**[001422]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid expansion is split into a plurality of steps to achieve a scaling out of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a first small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 2 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the first population of T cells from first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 5 to 7 days.

**[001423]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid expansion

is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 2 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 5 to 7 days.

**[001424]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 5 to 6 days.

**[001425]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 5 days.

**[001426]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small

scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 6 days.

**[001427]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing the first population of T cells in a small scale culture in a first container, *e.g.*, a G-REX 100MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the first population of T cells from the small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX 500MCS containers, wherein in each second container the portion of the first population of T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 7 days.

**[001428]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion of step (a) is performed during a period of up to 7 days.

**[001429]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the rapid second expansion of step (b) is performed during a period of up to 8 days.

**[001430]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the rapid second expansion of step (b) is performed during a period of up to 9 days.

**[001431]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the rapid second expansion of step (b) is performed during a period of up to 10 days.

**[001432]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the rapid second expansion of step (b) is performed during a period of up to 11 days.

**[001433]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion in step (a) is performed during a period of 7 days and the rapid second expansion of step (b) is performed during a period of up to 9 days.

**[001434]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion in step (a) is performed during a period of 7 days and the rapid second expansion of step (b) is performed during a period of up to 10 days.

**[001435]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion in step (a) is performed during a period of 7 days or 8 days and the rapid second expansion of step (b) is performed during a period of up to 9 days.

**[001436]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion in step (a) is performed during a period of 7 days or 8 days and the rapid second expansion of step (b) is performed during a period of up to 10 days.

**[001437]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion in step (a) is performed during a period of 8 days and the rapid second expansion of step (b) is performed during a period of up to 9 days.

**[001438]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the priming first expansion in step (a) is performed during a period of 8 days and the rapid second expansion of step (b) is performed during a period of up to 8 days.

**[001439]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of T cells is cultured in a first culture medium comprising OKT-3 and IL-2.

**[001440]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first culture medium comprises 4-1BB agonist, OKT-3 and IL-2.

**[001441]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first culture medium comprises OKT-3, IL-2 and antigen-presenting cells (APCs).

**[001442]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first culture medium comprises 4-1BB agonist, OKT-3, IL-2 and antigen-presenting cells (APCs).

**[001443]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the first population of T cells is cultured in a second culture medium comprising OKT-3, IL-2 and antigen-presenting cells (APCs).

**[001444]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the second culture medium comprises 4-1BB agonist, OKT-3, IL-2 and antigen-presenting cells (APCs).

**[001445]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of T cells is cultured in a first culture medium in a container comprising a first gas-permeable surface, wherein the first culture medium comprises OKT-3, IL-2 and a first population of antigen-presenting cells (APCs), wherein the first population of APCs is exogenous to the donor of the first population of T cells and the first population of APCs is layered onto the first gas-permeable surface, wherein in step (b) the first population of T cells is cultured in a second culture medium in the container, wherein the second culture medium comprises OKT-3, IL-2 and a second population of APCs, wherein the second population of APCs is exogenous to the donor of the first population of T cells and the second population of APCs is layered onto the first gas-permeable surface, and wherein the second population of APCs is greater than the first population of APCs.

**[001446]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of T cells is cultured in a first culture medium in a container comprising a first gas-permeable surface, wherein the first culture medium comprises 4-1BB agonist, OKT-3, IL-2 and a first population of antigen-presenting cells (APCs), wherein the first population of APCs is exogenous to the donor of the first population of T cells and the first population of APCs is layered onto the first gas-permeable surface, wherein in step (b) the first population

of T cells is cultured in a second culture medium in the container, wherein the second culture medium comprises OKT-3, IL-2 and a second population of APCs, wherein the second population of APCs is exogenous to the donor of the first population of T cells and the second population of APCs is layered onto the first gas-permeable surface, and wherein the second population of APCs is greater than the first population of APCs.

**[001447]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of T cells is cultured in a first culture medium in a container comprising a first gas-permeable surface, wherein the first culture medium comprises OKT-3, IL-2 and a first population of antigen-presenting cells (APCs), wherein the first population of APCs is exogenous to the donor of the first population of T cells and the first population of APCs is layered onto the first gas-permeable surface, wherein in step (b) the first population of T cells is cultured in a second culture medium in the container, wherein the second culture medium comprises 4-1BB agonist, OKT-3, IL-2 and a second population of APCs, wherein the second population of APCs is exogenous to the donor of the first population of T cells and the second population of APCs is layered onto the first gas-permeable surface, and wherein the second population of APCs is greater than the first population of APCs.

**[001448]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of T cells is cultured in a first culture medium in a container comprising a first gas-permeable surface, wherein the first culture medium comprises 4-1BB agonist, OKT-3, IL-2 and a first population of antigen-presenting cells (APCs), wherein the first population of APCs is exogenous to the donor of the first population of T cells and the first population of APCs is layered onto the first gas-permeable surface, wherein in step (b) the first population of T cells is cultured in a second culture medium in the container, wherein the second culture medium comprises 4-1BB agonist, OKT-3, IL-2 and a second population of APCs, wherein the second population of APCs is exogenous to the donor of the first population of T cells and the second population of APCs is layered onto the first gas-permeable surface, and wherein the second population of APCs is greater than the first population of APCs.

**[001449]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the number of APCs in the second population of APCs to the number of APCs in the first population of APCs is about 2:1.

**[001450]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the number of APCs in the first population of APCs is about 2.5 x $10^8$ and the number of APCs in the second population of APCs is about 5 x $10^8$.

**[001451]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is layered onto the first gas-permeable surface at an average thickness of 2 layers of APCs.

**[001452]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the second population of APCs is layered onto the first gas-permeable surface at an average thickness selected from the range of 4 to 8 layers of APCs.

**[001453]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the ratio of the average number of layers of APCs layered onto the first gas-permeable surface in step (b) to the average number of layers of APCs layered onto the first gas-permeable surface in step (a) is 2:1.

**[001454]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $1.0\times10^6$ APCs/$cm^2$ to at or about $4.5\times10^6$ APCs/$cm^2$.

**[001455]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $1.5\times10^6$ APCs/$cm^2$ to at or about $3.5\times10^6$ APCs/$cm^2$.

**[001456]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $2.0\times10^6$ APCs/$cm^2$ to at or about $3.0\times10^6$ APCs/$cm^2$.

**[001457]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density of at or about $2.0\times10^6$ APCs/$cm^2$.

**[001458]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the second population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $2.5\times10^6$ APCs/cm$^2$ to at or about $7.5\times10^6$ APCs/cm$^2$.

**[001459]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the second population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $3.5\times10^6$ APCs/cm$^2$ to at or about $6.0\times10^6$ APCs/cm$^2$.

**[001460]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the second population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $4.0\times10^6$ APCs/cm$^2$ to at or about $5.5\times10^6$ APCs/cm$^2$.

**[001461]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (b) the second population of APCs is seeded on the first gas permeable surface at a density of at or about $4.0\times10^6$ APCs/cm$^2$.

**[001462]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $1.0\times10^6$ APCs/cm$^2$ to at or about $4.5\times10^6$ APCs/cm$^2$ and in step (b) the second population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $2.5\times10^6$ APCs/cm$^2$ to at or about $7.5\times10^6$ APCs/cm$^2$.

**[001463]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $1.5\times10^6$ APCs/cm$^2$ to at or about $3.5\times10^6$ APCs/cm$^2$ and in step (b) the second population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $3.5\times10^6$ APCs/cm$^2$ to at or about $6.0\times10^6$ APCs/cm$^2$.

**[001464]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $2.0\times10^6$ APCs/cm$^2$ to at or about $3.0\times10^6$ APCs/cm$^2$ and in step (b) the second

population of APCs is seeded on the first gas permeable surface at a density selected from the range of at or about $4.0\times10^6$ APCs/$cm^2$ to at or about $5.5\times10^6$ APCs/$cm^2$.

**[001465]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that in step (a) the first population of APCs is seeded on the first gas permeable surface at a density of at or about $2.0\times10^6$ APCs/$cm^2$ and in step (b) the second population of APCs is seeded on the first gas permeable surface at a density of at or about $4.0\times10^6$ APCs/$cm^2$.

**[001466]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the APCs are peripheral blood mononuclear cells (PBMCs).

**[001467]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the PBMCs are irradiated and exogenous to the donor of the first population of T cells.

**[001468]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the T cells are tumor infiltrating lymphocytes (TILs).

**[001469]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the T cells are marrow infiltrating lymphocytes (MILs).

**[001470]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the T cells are peripheral blood lymphocytes (PBLs).

**[001471]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first population of T cells is obtained by separation from the whole blood of the donor.

**[001472]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first population of T cells is obtained by separation from the apheresis product of the donor.

**[001473]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first population of T cells is

separated from the whole blood or apheresis product of the donor by positive or negative selection of a T cell phenotype.

**[001474]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the T cell phenotype is CD3+ and CD45+.

**[001475]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that before performing the priming first expansion of the first population of T cells the T cells are separated from NK cells. In another embodiment, the T cells are separated from NK cells in the first population of T cells by removal of CD3- CD56+ cells from the first population of T cells. In another embodiment, the CD3- CD56+ cells are removed from the first population of T cells by subjecting the first population of T cells to cell sorting using a gating strategy that removes the CD3- CD56+ cell fraction and recovers the negative fraction. In another embodiment, the foregoing method is utilized for the expansion of T cells in a first population of T cells characterized by a high percentage of NK cells. In another embodiment, the foregoing method is utilized for the expansion of T cells in a first population of T cells characterized by a high percentage of CD3- CD56+ cells. In another embodiment, the foregoing method is utilized for the expansion of T cells in tumor tissue characterized by the present of a high number of NK cells. In another embodiment, the foregoing method is utilized for the expansion of T cells in tumor tissue characterized by a high number of CD3- CD56+ cells. In another embodiment, the foregoing method is utilized for the expansion of T cells in tumor tissue obtained from a patient suffering from a tumor characterized by the presence of a high number of NK cells. In another embodiment, the foregoing method is utilized for the expansion of T cells in tumor tissue obtained from a patient suffering from a tumor characterized by the presence of a high number of CD3- CD56+ cells. In another embodiment, the foregoing method is utilized for the expansion of T cells in tumor tissue obtained from a patient suffering from ovarian cancer.

**[001476]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that at or about $1x10^7$ T cells from the first population of T cells are seeded in a container to initiate the primary first expansion culture in such container.

**[001477]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the first population of T cells is distributed into a plurality of containers, and in each container at or about $1x10^7$ T cells from the first population of T cells are seeded to initiate the primary first expansion culture in such container.

**[001478]** In another embodiment, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that the second population of T cells harvested in step (c) is a therapeutic population of TILs.

## V. Pharmaceutical Compositions, Dosages, and Dosing Regimens

**[001479]** In an embodiment, TILs expanded using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In an embodiment, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded using PBMCs of the present disclosure may be administered by any suitable route as known in the art. In some embodiments, the T-cells are administered as a single intra-arterial or intravenous infusion, which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic administration.

**[001480]** Any suitable dose of TILs can be administered. In some embodiments, from about $2.3\times10^{10}$ to about $13.7\times10^{10}$ TILs are administered, with an average of around $7.8\times10^{10}$ TILs, particularly if the cancer is melanoma. In an embodiment, about $1.2\times10^{10}$ to about $4.3\times10^{10}$ of TILs are administered. In some embodiments, about $3\times10^{10}$ to about $12\times10^{10}$ TILs are administered. In some embodiments, about $4\times10^{10}$ to about $10\times10^{10}$ TILs are administered. In some embodiments, about $5\times10^{10}$ to about $8\times10^{10}$ TILs are administered. In some embodiments, about $6\times10^{10}$ to about $8\times10^{10}$ TILs are administered. In some embodiments, about $7\times10^{10}$ to about $8\times10^{10}$ TILs are administered. In some embodiments, the therapeutically effective dosage is about $2.3\times10^{10}$ to about $13.7\times10^{10}$. In some embodiments, the therapeutically effective dosage is about $7.8\times10^{10}$ TILs, particularly of the cancer is melanoma. In some embodiments, the therapeutically effective dosage is about $1.2\times10^{10}$ to about $4.3\times10^{10}$ of TILs. In some embodiments, the therapeutically effective dosage is about $3\times10^{10}$ to about $12\times10^{10}$ TILs. In some embodiments, the therapeutically effective dosage is about $4\times10^{10}$ to about $10\times10^{10}$ TILs. In some embodiments, the therapeutically effective

dosage is about $5\times10^{10}$ to about $8\times10^{10}$ TILs. In some embodiments, the therapeutically effective dosage is about $6\times10^{10}$ to about $8\times10^{10}$ TILs. In some embodiments, the therapeutically effective dosage is about $7\times10^{10}$ to about $8\times10^{10}$ TILs.

**[001481]** In some embodiments, the number of the TILs provided in the pharmaceutical compositions of the invention is about $1\times10^{6}$, $2\times10^{6}$, $3\times10^{6}$, $4\times10^{6}$, $5\times10^{6}$, $6\times10^{6}$, $7\times10^{6}$, $8\times10^{6}$, $9\times10^{6}$, $1\times10^{7}$, $2\times10^{7}$, $3\times10^{7}$, $4\times10^{7}$, $5\times10^{7}$, $6\times10^{7}$, $7\times10^{7}$, $8\times10^{7}$, $9\times10^{7}$, $1\times10^{8}$, $2\times10^{8}$, $3\times10^{8}$, $4\times10^{8}$, $5\times10^{8}$, $6\times10^{8}$, $7\times10^{8}$, $8\times10^{8}$, $9\times10^{8}$, $1\times10^{9}$, $2\times10^{9}$, $3\times10^{9}$, $4\times10^{9}$, $5\times10^{9}$, $6\times10^{9}$, $7\times10^{9}$, $8\times10^{9}$, $9\times10^{9}$, $1\times10^{10}$, $2\times10^{10}$, $3\times10^{10}$, $4\times10^{10}$, $5\times10^{10}$, $6\times10^{10}$, $7\times10^{10}$, $8\times10^{10}$, $9\times10^{10}$, $1\times10^{11}$, $2\times10^{11}$, $3\times10^{11}$, $4\times10^{11}$, $5\times10^{11}$, $6\times10^{11}$, $7\times10^{11}$, $8\times10^{11}$, $9\times10^{11}$, $1\times10^{12}$, $2\times10^{12}$, $3\times10^{12}$, $4\times10^{12}$, $5\times10^{12}$, $6\times10^{12}$, $7\times10^{12}$, $8\times10^{12}$, $9\times10^{12}$, $1\times10^{13}$, $2\times10^{13}$, $3\times10^{13}$, $4\times10^{13}$, $5\times10^{13}$, $6\times10^{13}$, $7\times10^{13}$, $8\times10^{13}$, and $9\times10^{13}$. In an embodiment, the number of the TILs provided in the pharmaceutical compositions of the invention is in the range of $1\times10^{6}$ to $5\times10^{6}$, $5\times10^{6}$ to $1\times10^{7}$, $1\times10^{7}$ to $5\times10^{7}$, $5\times10^{7}$ to $1\times10^{8}$, $1\times10^{8}$ to $5\times10^{8}$, $5\times10^{8}$ to $1\times10^{9}$, $1\times10^{9}$ to $5\times10^{9}$, $5\times10^{9}$ to $1\times10^{10}$, $1\times10^{10}$ to $5\times10^{10}$, $5\times10^{10}$ to $1\times10^{11}$, $5\times10^{11}$ to $1\times10^{12}$, $1\times10^{12}$ to $5\times10^{12}$, and $5\times10^{12}$ to $1\times10^{13}$.

**[001482]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is less than, for example, 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002% or 0.0001% w/w, w/v or v/v of the pharmaceutical composition.

**[001483]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is greater than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 19.75%, 19.50%, 19.25% 19%, 18.75%, 18.50%, 18.25% 18%, 17.75%, 17.50%, 17.25% 17%, 16.75%, 16.50%, 16.25% 16%, 15.75%, 15.50%, 15.25% 15%, 14.75%, 14.50%, 14.25% 14%, 13.75%, 13.50%, 13.25% 13%, 12.75%, 12.50%, 12.25% 12%, 11.75%, 11.50%, 11.25% 11%, 10.75%, 10.50%, 10.25% 10%, 9.75%, 9.50%, 9.25% 9%, 8.75%, 8.50%, 8.25% 8%, 7.75%, 7.50%, 7.25% 7%, 6.75%, 6.50%, 6.25% 6%, 5.75%, 5.50%, 5.25% 5%, 4.75%, 4.50%, 4.25%, 4%, 3.75%, 3.50%, 3.25%, 3%, 2.75%, 2.50%, 2.25%, 2%, 1.75%, 1.50%, 125%, 1%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%,

0.0004%, 0.0003%, 0.0002% or 0.0001% w/w, w/v, or v/v of the pharmaceutical composition.

**[001484]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is in the range from about 0.0001% to about 50%, about 0.001% to about 40%, about 0.01% to about 30%, about 0.02% to about 29%, about 0.03% to about 28%, about 0.04% to about 27%, about 0.05% to about 26%, about 0.06% to about 25%, about 0.07% to about 24%, about 0.08% to about 23%, about 0.09% to about 22%, about 0.1% to about 21%, about 0.2% to about 20%, about 0.3% to about 19%, about 0.4% to about 18%, about 0.5% to about 17%, about 0.6% to about 16%, about 0.7% to about 15%, about 0.8% to about 14%, about 0.9% to about 12% or about 1% to about 10% w/w, w/v or v/v of the pharmaceutical composition.

**[001485]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is in the range from about 0.001% to about 10%, about 0.01% to about 5%, about 0.02% to about 4.5%, about 0.03% to about 4%, about 0.04% to about 3.5%, about 0.05% to about 3%, about 0.06% to about 2.5%, about 0.07% to about 2%, about 0.08% to about 1.5%, about 0.09% to about 1%, about 0.1% to about 0.9% w/w, w/v or v/v of the pharmaceutical composition.

**[001486]** In some embodiments, the amount of the TILs provided in the pharmaceutical compositions of the invention is equal to or less than 10 g, 9.5 g, 9.0 g, 8.5 g, 8.0 g, 7.5 g, 7.0 g, 6.5 g, 6.0 g, 5.5 g, 5.0 g, 4.5 g, 4.0 g, 3.5 g, 3.0 g, 2.5 g, 2.0 g, 1.5 g, 1.0 g, 0.95 g, 0.9 g, 0.85 g, 0.8 g, 0.75 g, 0.7 g, 0.65 g, 0.6 g, 0.55 g, 0.5 g, 0.45 g, 0.4 g, 0.35 g, 0.3 g, 0.25 g, 0.2 g, 0.15 g, 0.1 g, 0.09 g, 0.08 g, 0.07 g, 0.06 g, 0.05 g, 0.04 g, 0.03 g, 0.02 g, 0.01 g, 0.009 g, 0.008 g, 0.007 g, 0.006 g, 0.005 g, 0.004 g, 0.003 g, 0.002 g, 0.001 g, 0.0009 g, 0.0008 g, 0.0007 g, 0.0006 g, 0.0005 g, 0.0004 g, 0.0003 g, 0.0002 g, or 0.0001 g.

**[001487]** In some embodiments, the amount of the TILs provided in the pharmaceutical compositions of the invention is more than 0.0001 g, 0.0002 g, 0.0003 g, 0.0004 g, 0.0005 g, 0.0006 g, 0.0007 g, 0.0008 g, 0.0009 g, 0.001 g, 0.0015 g, 0.002 g, 0.0025 g, 0.003 g, 0.0035 g, 0.004 g, 0.0045 g, 0.005 g, 0.0055 g, 0.006 g, 0.0065 g, 0.007 g, 0.0075 g, 0.008 g, 0.0085 g, 0.009 g, 0.0095 g, 0.01 g, 0.015 g, 0.02 g, 0.025 g, 0.03 g, 0.035 g, 0.04 g, 0.045 g, 0.05 g, 0.055 g, 0.06 g, 0.065 g, 0.07 g, 0.075 g, 0.08 g, 0.085 g, 0.09 g, 0.095 g, 0.1 g, 0.15 g, 0.2 g, 0.25 g, 0.3 g, 0.35 g, 0.4 g, 0.45 g, 0.5 g, 0.55 g, 0.6 g, 0.65 g, 0.7 g, 0.75 g, 0.8 g, 0.85 g, 0.9

g, 0.95 g, 1 g, 1.5 g, 2 g, 2.5, 3 g, 3.5, 4 g, 4.5 g, 5 g, 5.5 g, 6 g, 6.5 g, 7 g, 7.5 g, 8 g, 8.5 g, 9 g, 9.5 g, or 10 g.

**[001488]** The TILs provided in the pharmaceutical compositions of the invention are effective over a wide dosage range. The exact dosage will depend upon the route of administration, the form in which the compound is administered, the gender and age of the subject to be treated, the body weight of the subject to be treated, and the preference and experience of the attending physician. The clinically-established dosages of the TILs may also be used if appropriate. The amounts of the pharmaceutical compositions administered using the methods herein, such as the dosages of TILs, will be dependent on the human or mammal being treated, the severity of the disorder or condition, the rate of administration, the disposition of the active pharmaceutical ingredients and the discretion of the prescribing physician.

**[001489]** In some embodiments, TILs may be administered in a single dose. Such administration may be by injection, *e.g.*, intravenous injection. In some embodiments, TILs may be administered in multiple doses. Dosing may be once, twice, three times, four times, five times, six times, or more than six times per year. Dosing may be once a month, once every two weeks, once a week, or once every other day. Administration of TILs may continue as long as necessary.

**[001490]** In some embodiments, an effective dosage of TILs is about $1\times10^6$, $2\times10^6$, $3\times10^6$, $4\times10^6$, $5\times10^6$, $6\times10^6$, $7\times10^6$, $8\times10^6$, $9\times10^6$, $1\times10^7$, $2\times10^7$, $3\times10^7$, $4\times10^7$, $5\times10^7$, $6\times10^7$, $7\times10^7$, $8\times10^7$, $9\times10^7$, $1\times10^8$, $2\times10^8$, $3\times10^8$, $4\times10^8$, $5\times10^8$, $6\times10^8$, $7\times10^8$, $8\times10^8$, $9\times10^8$, $1\times10^9$, $2\times10^9$, $3\times10^9$, $4\times10^9$, $5\times10^9$, $6\times10^9$, $7\times10^9$, $8\times10^9$, $9\times10^9$, $1\times10^{10}$, $2\times10^{10}$, $3\times10^{10}$, $4\times10^{10}$, $5\times10^{10}$, $6\times10^{10}$, $7\times10^{10}$, $8\times10^{10}$, $9\times10^{10}$, $1\times10^{11}$, $2\times10^{11}$, $3\times10^{11}$, $4\times10^{11}$, $5\times10^{11}$, $6\times10^{11}$, $7\times10^{11}$, $8\times10^{11}$, $9\times10^{11}$, $1\times10^{12}$, $2\times10^{12}$, $3\times10^{12}$, $4\times10^{12}$, $5\times10^{12}$, $6\times10^{12}$, $7\times10^{12}$, $8\times10^{12}$, $9\times10^{12}$, $1\times10^{13}$, $2\times10^{13}$, $3\times10^{13}$, $4\times10^{13}$, $5\times10^{13}$, $6\times10^{13}$, $7\times10^{13}$, $8\times10^{13}$, and $9\times10^{13}$. In some embodiments, an effective dosage of TILs is in the range of $1\times10^6$ to $5\times10^6$, $5\times10^6$ to $1\times10^7$, $1\times10^7$ to $5\times10^7$, $5\times10^7$ to $1\times10^8$, $1\times10^8$ to $5\times10^8$, $5\times10^8$ to $1\times10^9$, $1\times10^9$ to $5\times10^9$, $5\times10^9$ to $1\times10^{10}$, $1\times10^{10}$ to $5\times10^{10}$, $5\times10^{10}$ to $1\times10^{11}$, $5\times10^{11}$ to $1\times10^{12}$, $1\times10^{12}$ to $5\times10^{12}$, and $5\times10^{12}$ to $1\times10^{13}$.

**[001491]** In some embodiments, an effective dosage of TILs is in the range of about 0.01 mg/kg to about 4.3 mg/kg, about 0.15 mg/kg to about 3.6 mg/kg, about 0.3 mg/kg to about 3.2 mg/kg, about 0.35 mg/kg to about 2.85 mg/kg, about 0.15 mg/kg to about 2.85 mg/kg,

about 0.3 mg to about 2.15 mg/kg, about 0.45 mg/kg to about 1.7 mg/kg, about 0.15 mg/kg to about 1.3 mg/kg, about 0.3 mg/kg to about 1.15 mg/kg, about 0.45 mg/kg to about 1 mg/kg, about 0.55 mg/kg to about 0.85 mg/kg, about 0.65 mg/kg to about 0.8 mg/kg, about 0.7 mg/kg to about 0.75 mg/kg, about 0.7 mg/kg to about 2.15 mg/kg, about 0.85 mg/kg to about 2 mg/kg, about 1 mg/kg to about 1.85 mg/kg, about 1.15 mg/kg to about 1.7 mg/kg, about 1.3 mg/kg mg to about 1.6 mg/kg, about 1.35 mg/kg to about 1.5 mg/kg, about 2.15 mg/kg to about 3.6 mg/kg, about 2.3 mg/kg to about 3.4 mg/kg, about 2.4 mg/kg to about 3.3 mg/kg, about 2.6 mg/kg to about 3.15 mg/kg, about 2.7 mg/kg to about 3 mg/kg, about 2.8 mg/kg to about 3 mg/kg, or about 2.85 mg/kg to about 2.95 mg/kg.

**[001492]** In some embodiments, an effective dosage of TILs is in the range of about 1 mg to about 500 mg, about 10 mg to about 300 mg, about 20 mg to about 250 mg, about 25 mg to about 200 mg, about 1 mg to about 50 mg, about 5 mg to about 45 mg, about 10 mg to about 40 mg, about 15 mg to about 35 mg, about 20 mg to about 30 mg, about 23 mg to about 28 mg, about 50 mg to about 150 mg, about 60 mg to about 140 mg, about 70 mg to about 130 mg, about 80 mg to about 120 mg, about 90 mg to about 110 mg, or about 95 mg to about 105 mg, about 98 mg to about 102 mg, about 150 mg to about 250 mg, about 160 mg to about 240 mg, about 170 mg to about 230 mg, about 180 mg to about 220 mg, about 190 mg to about 210 mg, about 195 mg to about 205 mg, or about 198 to about 207 mg.

**[001493]** An effective amount of the TILs may be administered in either single or multiple doses by any of the accepted modes of administration of agents having similar utilities, including intranasal and transdermal routes, by intra-arterial injection, intravenously, intraperitoneally, parenterally, intramuscularly, subcutaneously, topically, by transplantation, or by inhalation.

**[001494]** In another embodiment, the invention provides an infusion bag comprising the therapeutic population of TILs described in any of the preceding paragraphs above.

**[001495]** In another embodiment, the invention provides a tumor infiltrating lymphocyte (TIL) composition comprising the therapeutic population of TILs described in any of the preceding paragraphs above and a pharmaceutically acceptable carrier.

**[001496]** In another embodiment, the invention provides an infusion bag comprising the TIL composition described in any of the preceding paragraphs above.

**[001497]** In another embodiment, the invention provides a cryopreserved preparation of the therapeutic population of TILs described in any of the preceding paragraphs above.

**[001498]** In another embodiment, the invention provides a tumor infiltrating lymphocyte (TIL) composition comprising the therapeutic population of TILs described in any of the preceding paragraphs above and a cryopreservation media.

**[001499]** In another embodiment, the invention provides the TIL composition described in any of the preceding paragraphs above modified such that the cryopreservation media contains DMSO.

**[001500]** In another embodiment, the invention provides the TIL composition described in any of the preceding paragraphs above modified such that the cryopreservation media contains 7-10% DMSO.

**[001501]** In another embodiment, the invention provides a cryopreserved preparation of the TIL composition described in any of the preceding paragraphs above.

## VI. Pharmaceutical Compositions, Dosages, and Dosing Regimens

**[001502]** In an embodiment, TILs expanded using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In an embodiment, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded using PBMCs of the present disclosure may be administered by any suitable route as known in the art. In some embodiments, the T-cells are administered as a single intra-arterial or intravenous infusion, which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic administration.

**[001503]** Any suitable dose of TILs can be administered. In some embodiments, from about $2.3\times10^{10}$ to about $13.7\times10^{10}$ TILs are administered, with an average of around $7.8\times10^{10}$ TILs, particularly if the cancer is NSCLC. In an embodiment, about $1.2\times10^{10}$ to about $4.3\times10^{10}$ of TILs are administered. In some embodiments, about $3\times10^{10}$ to about $12\times10^{10}$ TILs are administered. In some embodiments, about $4\times10^{10}$ to about $10\times10^{10}$ TILs are administered. In some embodiments, about $5\times10^{10}$ to about $8\times10^{10}$ TILs are administered. In some embodiments, about $6\times10^{10}$ to about $8\times10^{10}$ TILs are administered. In some embodiments, about $7\times10^{10}$ to about $8\times10^{10}$ TILs are administered. In some embodiments, therapeutically effective dosage is about $2.3\times10^{10}$ to about $13.7\times10^{10}$. In some embodiments, therapeutically effective dosage is about $7.8\times10^{10}$ TILs, particularly of the cancer is NSCLC. In some embodiments, therapeutically effective dosage is about $1.2\times10^{10}$ to about $4.3\times10^{10}$ of TILs. In some embodiments, therapeutically effective dosage is about $3\times10^{10}$ to about $12\times10^{10}$ TILs.

In some embodiments, therapeutically effective dosage is about $4\times10^{10}$ to about $10\times10^{10}$ TILs. In some embodiments, therapeutically effective dosage is about $5\times10^{10}$ to about $8\times10^{10}$ TILs. In some embodiments, therapeutically effective dosage is about $6\times10^{10}$ to about $8\times10^{10}$ TILs. In some embodiments, therapeutically effective dosage is about $7\times10^{10}$ to about $8\times10^{10}$ TILs.

**[001504]** In some embodiments, the number of the TILs provided in the pharmaceutical compositions of the invention is about $1\times10^{6}$, $2\times10^{6}$, $3\times10^{6}$, $4\times10^{6}$, $5\times10^{6}$, $6\times10^{6}$, $7\times10^{6}$, $8\times10^{6}$, $9\times10^{6}$, $1\times10^{7}$, $2\times10^{7}$, $3\times10^{7}$, $4\times10^{7}$, $5\times10^{7}$, $6\times10^{7}$, $7\times10^{7}$, $8\times10^{7}$, $9\times10^{7}$, $1\times10^{8}$, $2\times10^{8}$, $3\times10^{8}$, $4\times10^{8}$, $5\times10^{8}$, $6\times10^{8}$, $7\times10^{8}$, $8\times10^{8}$, $9\times10^{8}$, $1\times10^{9}$, $2\times10^{9}$, $3\times10^{9}$, $4\times10^{9}$, $5\times10^{9}$, $6\times10^{9}$, $7\times10^{9}$, $8\times10^{9}$, $9\times10^{9}$, $1\times10^{10}$, $2\times10^{10}$, $3\times10^{10}$, $4\times10^{10}$, $5\times10^{10}$, $6\times10^{10}$, $7\times10^{10}$, $8\times10^{10}$, $9\times10^{10}$, $1\times10^{11}$, $2\times10^{11}$, $3\times10^{11}$, $4\times10^{11}$, $5\times10^{11}$, $6\times10^{11}$, $7\times10^{11}$, $8\times10^{11}$, $9\times10^{11}$, $1\times10^{12}$, $2\times10^{12}$, $3\times10^{12}$, $4\times10^{12}$, $5\times10^{12}$, $6\times10^{12}$, $7\times10^{12}$, $8\times10^{12}$, $9\times10^{12}$, $1\times10^{13}$, $2\times10^{13}$, $3\times10^{13}$, $4\times10^{13}$, $5\times10^{13}$, $6\times10^{13}$, $7\times10^{13}$, $8\times10^{13}$, and $9\times10^{13}$. In an embodiment, the number of the TILs provided in the pharmaceutical compositions of the invention is in the range of $1\times10^{6}$ to $5\times10^{6}$, $5\times10^{6}$ to $1\times10^{7}$, $1\times10^{7}$ to $5\times10^{7}$, $5\times10^{7}$ to $1\times10^{8}$, $1\times10^{8}$ to $5\times10^{8}$, $5\times10^{8}$ to $1\times10^{9}$, $1\times10^{9}$ to $5\times10^{9}$, $5\times10^{9}$ to $1\times10^{10}$, $1\times10^{10}$ to $5\times10^{10}$, $5\times10^{10}$ to $1\times10^{11}$, $5\times10^{11}$ to $1\times10^{12}$, $1\times10^{12}$ to $5\times10^{12}$, and $5\times10^{12}$ to $1\times10^{13}$.

**[001505]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is less than, for example, 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002% or 0.0001% w/w, w/v or v/v of the pharmaceutical composition.

**[001506]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is greater than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 19.75%, 19.50%, 19.25% 19%, 18.75%, 18.50%, 18.25% 18%, 17.75%, 17.50%, 17.25% 17%, 16.75%, 16.50%, 16.25% 16%, 15.75%, 15.50%, 15.25% 15%, 14.75%, 14.50%, 14.25% 14%, 13.75%, 13.50%, 13.25% 13%, 12.75%, 12.50%, 12.25% 12%, 11.75%, 11.50%, 11.25% 11%, 10.75%, 10.50%, 10.25% 10%, 9.75%, 9.50%, 9.25% 9%, 8.75%, 8.50%, 8.25% 8%, 7.75%, 7.50%, 7.25% 7%, 6.75%, 6.50%, 6.25% 6%, 5.75%, 5.50%, 5.25% 5%, 4.75%, 4.50%, 4.25%, 4%, 3.75%, 3.50%, 3.25%, 3%, 2.75%, 2.50%, 2.25%, 2%, 1.75%, 1.50%, 125%, 1%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1%, 0.09%, 0.08%, 0.07%,

0.06%, 0.05%, 0.04%, 0.03%, 0.02%, 0.01%, 0.009%, 0.008%, 0.007%, 0.006%, 0.005%, 0.004%, 0.003%, 0.002%, 0.001%, 0.0009%, 0.0008%, 0.0007%, 0.0006%, 0.0005%, 0.0004%, 0.0003%, 0.0002% or 0.0001% w/w, w/v, or v/v of the pharmaceutical composition.

**[001507]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is in the range from about 0.0001% to about 50%, about 0.001% to about 40%, about 0.01% to about 30%, about 0.02% to about 29%, about 0.03% to about 28%, about 0.04% to about 27%, about 0.05% to about 26%, about 0.06% to about 25%, about 0.07% to about 24%, about 0.08% to about 23%, about 0.09% to about 22%, about 0.1% to about 21%, about 0.2% to about 20%, about 0.3% to about 19%, about 0.4% to about 18%, about 0.5% to about 17%, about 0.6% to about 16%, about 0.7% to about 15%, about 0.8% to about 14%, about 0.9% to about 12% or about 1% to about 10% w/w, w/v or v/v of the pharmaceutical composition.

**[001508]** In some embodiments, the concentration of the TILs provided in the pharmaceutical compositions of the invention is in the range from about 0.001% to about 10%, about 0.01% to about 5%, about 0.02% to about 4.5%, about 0.03% to about 4%, about 0.04% to about 3.5%, about 0.05% to about 3%, about 0.06% to about 2.5%, about 0.07% to about 2%, about 0.08% to about 1.5%, about 0.09% to about 1%, about 0.1% to about 0.9% w/w, w/v or v/v of the pharmaceutical composition.

**[001509]** In some embodiments, the amount of the TILs provided in the pharmaceutical compositions of the invention is equal to or less than 10 g, 9.5 g, 9.0 g, 8.5 g, 8.0 g, 7.5 g, 7.0 g, 6.5 g, 6.0 g, 5.5 g, 5.0 g, 4.5 g, 4.0 g, 3.5 g, 3.0 g, 2.5 g, 2.0 g, 1.5 g, 1.0 g, 0.95 g, 0.9 g, 0.85 g, 0.8 g, 0.75 g, 0.7 g, 0.65 g, 0.6 g, 0.55 g, 0.5 g, 0.45 g, 0.4 g, 0.35 g, 0.3 g, 0.25 g, 0.2 g, 0.15 g, 0.1 g, 0.09 g, 0.08 g, 0.07 g, 0.06 g, 0.05 g, 0.04 g, 0.03 g, 0.02 g, 0.01 g, 0.009 g, 0.008 g, 0.007 g, 0.006 g, 0.005 g, 0.004 g, 0.003 g, 0.002 g, 0.001 g, 0.0009 g, 0.0008 g, 0.0007 g, 0.0006 g, 0.0005 g, 0.0004 g, 0.0003 g, 0.0002 g, or 0.0001 g.

**[001510]** In some embodiments, the amount of the TILs provided in the pharmaceutical compositions of the invention is more than 0.0001 g, 0.0002 g, 0.0003 g, 0.0004 g, 0.0005 g, 0.0006 g, 0.0007 g, 0.0008 g, 0.0009 g, 0.001 g, 0.0015 g, 0.002 g, 0.0025 g, 0.003 g, 0.0035 g, 0.004 g, 0.0045 g, 0.005 g, 0.0055 g, 0.006 g, 0.0065 g, 0.007 g, 0.0075 g, 0.008 g, 0.0085 g, 0.009 g, 0.0095 g, 0.01 g, 0.015 g, 0.02 g, 0.025 g, 0.03 g, 0.035 g, 0.04 g, 0.045 g, 0.05 g, 0.055 g, 0.06 g, 0.065 g, 0.07 g, 0.075 g, 0.08 g, 0.085 g, 0.09 g, 0.095 g, 0.1 g, 0.15 g, 0.2 g,

0.25 g, 0.3 g, 0.35 g, 0.4 g, 0.45 g, 0.5 g, 0.55 g, 0.6 g, 0.65 g, 0.7 g, 0.75 g, 0.8 g, 0.85 g, 0.9 g, 0.95 g, 1 g, 1.5 g, 2 g, 2.5, 3 g, 3.5, 4 g, 4.5 g, 5 g, 5.5 g, 6 g, 6.5 g, 7 g, 7.5 g, 8 g, 8.5 g, 9 g, 9.5 g, or 10 g.

**[001511]** The TILs provided in the pharmaceutical compositions of the invention are effective over a wide dosage range. The exact dosage will depend upon the route of administration, the form in which the compound is administered, the gender and age of the subject to be treated, the body weight of the subject to be treated, and the preference and experience of the attending physician. The clinically-established dosages of the TILs may also be used if appropriate. The amounts of the pharmaceutical compositions administered using the methods herein, such as the dosages of TILs, will be dependent on the human or mammal being treated, the severity of the disorder or condition, the rate of administration, the disposition of the active pharmaceutical ingredients and the discretion of the prescribing physician.

**[001512]** In some embodiments, TILs may be administered in a single dose. Such administration may be by injection, *e.g.*, intravenous injection. In some embodiments, TILs may be administered in multiple doses. Dosing may be once, twice, three times, four times, five times, six times, or more than six times per year. Dosing may be once a month, once every two weeks, once a week, or once every other day. Administration of TILs may continue as long as necessary.

**[001513]** In some embodiments, an effective dosage of TILs is about $1\times10^6$, $2\times10^6$, $3\times10^6$, $4\times10^6$, $5\times10^6$, $6\times10^6$, $7\times10^6$, $8\times10^6$, $9\times10^6$, $1\times10^7$, $2\times10^7$, $3\times10^7$, $4\times10^7$, $5\times10^7$, $6\times10^7$, $7\times10^7$, $8\times10^7$, $9\times10^7$, $1\times10^8$, $2\times10^8$, $3\times10^8$, $4\times10^8$, $5\times10^8$, $6\times10^8$, $7\times10^8$, $8\times10^8$, $9\times10^8$, $1\times10^9$, $2\times10^9$, $3\times10^9$, $4\times10^9$, $5\times10^9$, $6\times10^9$, $7\times10^9$, $8\times10^9$, $9\times10^9$, $1\times10^{10}$, $2\times10^{10}$, $3\times10^{10}$, $4\times10^{10}$, $5\times10^{10}$, $6\times10^{10}$, $7\times10^{10}$, $8\times10^{10}$, $9\times10^{10}$, $1\times10^{11}$, $2\times10^{11}$, $3\times10^{11}$, $4\times10^{11}$, $5\times10^{11}$, $6\times10^{11}$, $7\times10^{11}$, $8\times10^{11}$, $9\times10^{11}$, $1\times10^{12}$, $2\times10^{12}$, $3\times10^{12}$, $4\times10^{12}$, $5\times10^{12}$, $6\times10^{12}$, $7\times10^{12}$, $8\times10^{12}$, $9\times10^{12}$, $1\times10^{13}$, $2\times10^{13}$, $3\times10^{13}$, $4\times10^{13}$, $5\times10^{13}$, $6\times10^{13}$, $7\times10^{13}$, $8\times10^{13}$, and $9\times10^{13}$. In some embodiments, an effective dosage of TILs is in the range of $1\times10^6$ to $5\times10^6$, $5\times10^6$ to $1\times10^7$, $1\times10^7$ to $5\times10^7$, $5\times10^7$ to $1\times10^8$, $1\times10^8$ to $5\times10^8$, $5\times10^8$ to $1\times10^9$, $1\times10^9$ to $5\times10^9$, $5\times10^9$ to $1\times10^{10}$, $1\times10^{10}$ to $5\times10^{10}$, $5\times10^{10}$ to $1\times10^{11}$, $5\times10^{11}$ to $1\times10^{12}$, $1\times10^{12}$ to $5\times10^{12}$, and $5\times10^{12}$ to $1\times10^{13}$.

**[001514]** In some embodiments, an effective dosage of TILs is in the range of about 0.01 mg/kg to about 4.3 mg/kg, about 0.15 mg/kg to about 3.6 mg/kg, about 0.3 mg/kg to about

3.2 mg/kg, about 0.35 mg/kg to about 2.85 mg/kg, about 0.15 mg/kg to about 2.85 mg/kg, about 0.3 mg to about 2.15 mg/kg, about 0.45 mg/kg to about 1.7 mg/kg, about 0.15 mg/kg to about 1.3 mg/kg, about 0.3 mg/kg to about 1.15 mg/kg, about 0.45 mg/kg to about 1 mg/kg, about 0.55 mg/kg to about 0.85 mg/kg, about 0.65 mg/kg to about 0.8 mg/kg, about 0.7 mg/kg to about 0.75 mg/kg, about 0.7 mg/kg to about 2.15 mg/kg, about 0.85 mg/kg to about 2 mg/kg, about 1 mg/kg to about 1.85 mg/kg, about 1.15 mg/kg to about 1.7 mg/kg, about 1.3 mg/kg mg to about 1.6 mg/kg, about 1.35 mg/kg to about 1.5 mg/kg, about 2.15 mg/kg to about 3.6 mg/kg, about 2.3 mg/kg to about 3.4 mg/kg, about 2.4 mg/kg to about 3.3 mg/kg, about 2.6 mg/kg to about 3.15 mg/kg, about 2.7 mg/kg to about 3 mg/kg, about 2.8 mg/kg to about 3 mg/kg, or about 2.85 mg/kg to about 2.95 mg/kg.

**[001515]** In some embodiments, an effective dosage of TILs is in the range of about 1 mg to about 500 mg, about 10 mg to about 300 mg, about 20 mg to about 250 mg, about 25 mg to about 200 mg, about 1 mg to about 50 mg, about 5 mg to about 45 mg, about 10 mg to about 40 mg, about 15 mg to about 35 mg, about 20 mg to about 30 mg, about 23 mg to about 28 mg, about 50 mg to about 150 mg, about 60 mg to about 140 mg, about 70 mg to about 130 mg, about 80 mg to about 120 mg, about 90 mg to about 110 mg, or about 95 mg to about 105 mg, about 98 mg to about 102 mg, about 150 mg to about 250 mg, about 160 mg to about 240 mg, about 170 mg to about 230 mg, about 180 mg to about 220 mg, about 190 mg to about 210 mg, about 195 mg to about 205 mg, or about 198 to about 207 mg.

**[001516]** An effective amount of the TILs may be administered in either single or multiple doses by any of the accepted modes of administration of agents having similar utilities, including intranasal and transdermal routes, by intra-arterial injection, intravenously, intraperitoneally, parenterally, intramuscularly, subcutaneously, topically, by transplantation, or by inhalation.

## VII. Methods of Treating Patients

**[001517]** Methods of treatment begin with the initial TIL collection and culture of TILs. Such methods have been both described in the art by, for example, Jin *et al., J. Immunotherapy*, **2012**, 35(3):283-292, incorporated by reference herein in its entirety. Embodiments of methods of treatment are described throughout the sections below, including the Examples.

**[001518]** The expanded TILs produced according the methods described herein, including for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 1) find particular use in the treatment of patients with

cancer (for example, as described in Goff, et al., *J. Clinical Oncology*, **2016**, 34(20):2389-239, as well as the supplemental content; incorporated by reference herein in its entirety. In some embodiments, TIL were grown from resected deposits of metastatic melanoma as previously described (see, Dudley, et al., *J Immunother.*, **2003**, 26:332-342; incorporated by reference herein in its entirety). Fresh tumor can be dissected under sterile conditions. A representative sample can be collected for formal pathologic analysis. Single fragments of 2 $mm^3$ to 3 $mm^3$ may be used. In some embodiments, 5, 10, 15, 20, 25 or 30 samples per patient are obtained. In some embodiments, 20, 25, or 30 samples per patient are obtained. In some embodiments, 20, 22, 24, 26, or 28 samples per patient are obtained. In some embodiments, 24 samples per patient are obtained. Samples can be placed in individual wells of a 24-well plate, maintained in growth media with high-dose IL-2 (6,000 IU/mL), and monitored for destruction of tumor and/or proliferation of TIL. Any tumor with viable cells remaining after processing can be enzymatically digested into a single cell suspension and cryopreserved, as described herein.

**[001519]** In some embodiments, successfully grown TIL can be sampled for phenotype analysis (CD3, CD4, CD8, and CD56) and tested against autologous tumor when available. TIL can be considered reactive if overnight coculture yielded interferon-gamma (IFN-γ) levels > 200 pg/mL and twice background. (Goff, et al., *J Immunother.*, **2010**, 33:840-847; incorporated by reference herein in its entirety). In some embodiments, cultures with evidence of autologous reactivity or sufficient growth patterns can be selected for a second expansion (for example, a second expansion as provided in according to Step D of Figure 1), including second expansions that are sometimes referred to as rapid expansion (REP). In some embodiments, expanded TILs with high autologous reactivity (for example, high proliferation during a second expansion), are selected for an additional second expansion. In some embodiments, TILs with high autologous reactivity (for example, high proliferation during second expansion as provided in Step D of Figure 1), are selected for an additional second expansion according to Step D of Figure 1.

**[001520]** Cell phenotypes of cryopreserved samples of infusion bag TIL can be analyzed by flow cytometry (*e.g.,* FlowJo) for surface markers CD3, CD4, CD8, CCR7, and CD45RA (BD BioSciences), as well as by any of the methods described herein. Serum cytokines were measured by using standard enzyme-linked immunosorbent assay techniques. A rise in serum IFN-g was defined as >100 pg/mL and greater than 4 3 baseline levels.

**[001521]** In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 1, provide for a surprising improvement in clinical efficacy of the TILs. In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 1, exhibit increased clinical efficacy as compared to TILs produced by methods other than those described herein, including for example, methods other than those exemplified in Figure 1. In some embodiments, the methods other than those described herein include methods referred to as process 1C and/or Generation 1 (Gen 1). In some embodiments, the increased efficacy is measured by DCR, ORR, and/or other clinical responses. In some embodiments, the TILS produced by the methods provided herein, for example those exemplified in Figure 1, exhibit a similar time to response and safety profile compared to TILs produced by methods other than those described herein, including for example, methods other than those exemplified in Figure 1, for example the Gen 1 process.

**[001522]** In some embodiments, IFN-gamma (IFN-γ) is indicative of treatment efficacy and/or increased clinical efficacy. In some embodiments, IFN-γ in the blood of subjects treated with TILs is indicative of active TILs. In some embodiments, a potency assay for IFN-γ production is employed. IFN-γ production is another measure of cytotoxic potential. IFN-γ production can be measured by determining the levels of the cytokine IFN-γ in the blood, serum, or TILs *ex vivo* of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1. In some embodiments, an increase in IFN-γ is indicative of treatment efficacy in a patient treated with the TILs produced by the methods of the present invention. In some embodiments, IFN-γ is increased one-fold, two-fold, three-fold, four-fold, or five-fold or more as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN-γ secretion is increased one-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN-γ secretion is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN-γ secretion is increased three-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other

methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN-γ secretion is increased four-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN-γ secretion is increased five-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN-γ is measured using a Quantikine ELISA kit. In some embodiments, IFN-γ is measured in TILs *ex vivo* of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1. In some embodiments, IFN-γ is measured in blood of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1. In some embodiments, IFN-γ is measured in TILs serum of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1.

**[001523]** In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 1, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 1, such as for example, methods referred to as process 1C methods. In some embodiments, significantly improved polyclonality and/or increased polyclonality is indicative of treatment efficacy and/or increased clinical efficacy. In some embodiments, polyclonality refers to the T-cell repertoire diversity. In some embodiments, an increase in polyclonality can be indicative of treatment efficacy with regard to administration of the TILs produced by the methods of the present invention. In some embodiments, polyclonality is increased one-fold, two-fold, ten-fold, 100-fold, 500-fold, or 1000-fold as compared to TILs prepared using methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased one-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased ten-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other

methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 100-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 500-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 1000-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1.

**[001524]** Measures of efficacy can include the disease control rate (DCR) as well as overall response rate (ORR), as known in the art as well as described herein.

### 1. Methods of Treating NSCLC

**[001525]** The compositions and methods described herein can be used in a method for treating non-small-cell lung cancer (NSCLC), wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody. In some embodiments the anit-PD-1 antibody includes, *e.g.*, but is not limited to nivolumab (BMS-936558, Bristol-Myers Squibb; Opdivo®), pembrolizumab (lambrolizumab, MK03475 or MK-3475, Merck; Keytruda®), humanized anti-PD-1 antibody JS001 (ShangHai JunShi), monoclonal anti-PD-1 antibody TSR-042 (Tesaro, Inc.), Pidilizumab (anti-PD-1 mAb CT-011, Medivation), anti-PD-1 monoclonal Antibody BGB-A317 (BeiGene), and/or anti-PD-1 antibody SHR-1210 (ShangHai HengRui), human monoclonal antibody REGN2810 (Regeneron), human monoclonal antibody MDX-1106 (Bristol-Myers Squibb), and/or humanized anti-PD-1 IgG4 antibody PDR001 (Novartis). In some embodiments, the PD-1 antibody is from clone: RMP1-14 (rat IgG) - BioXcell cat# BP0146. Other suitable antibodies suitable for use in co-administration methods with TILs produced according to Steps A through F as described herein are anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, herein incorporated by reference. In some embodiments, the antibody or antigen-binding portion thereof binds specifically to PD-L1 and inhibits its interaction with PD-1, thereby increasing immune activity. Any antibodies known in the art which bind to PD-L1 and disrupt the interaction between the PD-1 and PD-L1, and stimulates an anti-tumor immu2740274ne response, are include. For example,

antibodies that target PD-L1 and are in clinical trials, include BMS-936559 (Bristol-Myers Squibb) and MPDL3280A (Genentech). Other suitable antibodies that target PD-L1 are disclosed in U.S. Patent No. 7,943,743, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to PD-1 or PD-L1, disrupts the PD-1/PD-L1 interaction, and stimulates an anti-tumor immune response, are included.

**[001526]** In some embodiments, the NSCLC is refractory to the anti-CTLA-4 and/or anti-PD-1 and a chemotherapeutic agent. In some embodiments, the NSCLC is refractory to the anti-CTLA-4 and/or anti-PD-1 and chemotherapy, wherein the chemotherapeutic agent is carboplatin, paclitaxel, pemetrexed, cisplatin. In some embodiments, the anti-CLTA-4 antibody, such as ipilimumab (Yervoy®).

**[001527]** In some embodiments, the NSCLC is refractory to treatment with combined PD-1 (including for example pembrolizumab) and a chemotherapeutic agent. In some embodiments, the chemotherapeutic agent(s) is a platinum doublet chemotherapeutic agent. In some embodiments, the platinum doublet therapy comprises a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin and a second chemotherapeutic agent selected from the group consisting of vinorelbine, gemcitabine and a taxane (including for example, paclitaxel, docetaxel or nab-paclitaxel). In some embodiments, the platinum doublet chemotherapeutic agent is in combination with pemetrexed.

**[001528]** In some embodiments, the NSCLC is refractory to a combination treatment or combination therapy comprising an anti-PD-1 and a chemotherapeutic agent. In some embodiments, the anti-PD-1 or the anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof. In some embodiments, the anti-PD-1 is pembrolizumab. In some embodiments, the chemotherapeutic agent is a platinum doublet chemotherapeutic agent. In some embodiments, the chemotherapeutic agent is in combination with pemetrexed. In some embodiments, the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, and cisplatin. In some embodiments, the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, cisplatin, nivolumab, and ipilimumab.

**[001529]** In some embodiments, the NSCLC is refractory to a combination therapy comprising the anti-PD-1 or the anti-PD-L1 and a platinum doublet therapy, wherein the platinum doublet therapy comprises:

i) a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin,

ii) and a second chemotherapeutic agent selected from the group consisting of vinorelbine, gemcitabine and a taxane (including for example, paclitaxel, docetaxel or nab-paclitaxel).

**[001530]** In some embodiments, the NSCLC is refractory to a combination therapy comprising the anti-PD-1 or the anti-PD-L1, pemetrexed, and a platinum doublet therapy, wherein the platinum doublet therapy comprises:

i) a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin,

ii) and a second chemotherapeutic agent selected from the group consisting of vinorelbine, gemcitabine and a taxane (including for example, paclitaxel, docetaxel or nab-paclitaxel).

**[001531]** In some embodiments, the NSCLC has been treated with an anti-PD-1 antibody. In some embodiments, the NSCLC has been treated with an anti-PD-L1 antibody. In some embodiments, the NSCLC subject is treatment naïve. In some embodiments, the NSCLC has not been treated with an anti-PD-1 antibody. In some embodiments, the NSCLC has not been treated with an anti-PD-L1 antibody. In some embodiments, the NSCLC has been previously treated with a chemotherapeutic agent. In some embodiments, the NSCLC has been previously treated with a chemotherapeutic agent but is not longer being treated with the chemotherapeutic agent. In some embodiments, the NSCLC patient is anti-PD-1/PD-L1 naïve. In some embodiments, the NSCLC subject has low expression of PD-L1. In some embodiments, the NSCLC subject has treatment naïve NSCLC or is post-chemotherapeutic treatment but anti-PD-1/PD-L1 naïve. In some embodiments, the NSCLC subject ist reatment naïve NSCLC or post-chemotherapuetic treament but anti-PD-1/PD-L1 naïve and has low expression of PD-L1. In some embodiments, the NSCLC subject has bulky disease at baseline. In some embodiments, the subject has bulky disease at baseline and has low expression of PD-L1. In some embodiments, the NSCLC subject has treatment naïve NSCLC or post chemotherapy (e.g., post chemotherapeutic agent) but anti-PD-1/PD-L1 naïve

who have low expression of PD-L1 and/or have bulky disease at baseline. In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane. In some embodiments, bulky disease is indicated when there are swollen lymph nodes with a short-axis diameter of 20 mm or greater. In some embodiments, the chemotherapeutic includes a standard of care therapeutic for NSCLC.

**[001532]** In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 1, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 1, such as for example, methods referred to as process 1C methods. In some embodiments, significantly improved polyclonality and/or increased polyclonality is indicative of treatment efficacy and/or increased clinical efficacy for cancer treatment. In some embodiments, polyclonality refers to the T-cell repertoire diversity. In some embodiments, an increase in polyclonality can be indicative of treatment efficacy with regard to administration of the TILs produced by the methods of the present invention. In some embodiments, polyclonality is increased one-fold, two-fold, ten-fold, 100-fold, 500-fold, or 1000-fold as compared to TILs prepared using methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased one-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased ten-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 100-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 500-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 1000-fold as

compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1.

a. PD-1 and PD-L1 Inhibitors

**[0001]** Programmed death 1 (PD-1) is a 288-amino acid transmembrane immunocheckpoint receptor protein expressed by T cells, B cells, natural killer (NK) T cells, activated monocytes, and dendritic cells. PD-1, which is also known as CD279, belongs to the CD28 family, and in humans is encoded by the *Pdcd1* gene on chromosome 2. PD-1 consists of one immunoglobulin (Ig) superfamily domain, a transmembrane region, and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 and its ligands (PD-L1 and PD-L2) are known to play a key role in immune tolerance, as described in Keir, *et al.*, *Annu. Rev. Immunol.* **2008,** *26,* 677-704. PD-1 provides inhibitory signals that negatively regulate T cell immune responses. PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) are expressed on tumor cells and stromal cells, which may be encountered by activated T cells expressing PD-1, leading to immunosuppression of the T cells. PD-L1 is a 290 amino acid transmembrane protein encoded by the *Cd274* gene on human chromosome 9. Blocking the interaction between PD-1 and its ligands PD-L1 and PD-L2 by use of a PD-1 inhibitor, a PD-L1 inhibitor, and/or a PD-L2 inhibitor can overcome immune resistance, as demonstrated in recent clinical studies, such as that described in Topalian, *et al.*, *N. Eng. J. Med.* **2012,** *366,* 2443-54. PD-L1 is expressed on many tumor cell lines, while PD-L2 is expressed is expressed mostly on dendritic cells and a few tumor lines. In addition to T cells (which inducibly express PD-1 after activation), PD-1 is also expressed on B cells, natural killer cells, macrophages, activated monocytes, and dendritic cells.

**[0002]** In an embodiment, the PD-1 inhibitor may be any PD-1 inhibitor or PD-1 blocker known in the art. In particular, it is one of the PD-1 inhibitors or blockers described in more detail in the following paragraphs. The terms "inhibitor," "antagonist," and "blocker" are used interchangeably herein in reference to PD-1 inhibitors. For avoidance of doubt, references herein to a PD-1 inhibitor that is an antibody may refer to a compound or antigen-binding fragments, variants, conjugates, or biosimilars thereof. For avoidance of doubt, references herein to a PD-1 inhibitor may also refer to a small molecule compound or a pharmaceutically acceptable salt, ester, solvate, hydrate, cocrystal, or prodrug thereof.

**[0003]** In a preferred embodiment, the PD-1 inhibitor is an antibody (*i.e.*, an anti-PD-1 antibody), a fragment thereof, including Fab fragments, or a single-chain variable fragment (scFv) thereof. In some embodiments the PD-1 inhibitor is a polyclonal antibody. In a preferred embodiment, the PD-1 inhibitor is a monoclonal antibody. In some embodiments, the PD-1 inhibitor competes for binding with PD-1, and/or binds to an epitope on PD-1. In an embodiment, the antibody competes for binding with PD-1, and/or binds to an epitope on PD-1.

**[0004]** In some embodiments, the PD-1 inhibitor is one that binds human PD-1 with a $K_D$ of about 100 pM or lower, binds human PD-1 with a $K_D$ of about 90 pM or lower, binds human PD-1 with a $K_D$ of about 80 pM or lower, binds human PD-1 with a $K_D$ of about 70 pM or lower, binds human PD-1 with a $K_D$ of about 60 pM or lower, binds human PD-1 with a $K_D$ of about 50 pM or lower, binds human PD-1 with a $K_D$ of about 40 pM or lower, binds human PD-1 with a $K_D$ of about 30 pM or lower, binds human PD-1 with a $K_D$ of about 20 pM or lower, binds human PD-1 with a $K_D$ of about 10 pM or lower, or binds human PD-1 with a $K_D$ of about 1 pM or lower.

**[0005]** In some embodiments, the PD-1 inhibitor is one that binds to human PD-1 with a $k_{assoc}$ of about $7.5 \times 10^5$ l/M·s or faster, binds to human PD-1 with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human PD-1 with a $k_{assoc}$ of about $8 \times 10^5$ 1/M·s or faster, binds to human PD-1 with a $k_{assoc}$ of about $8.5 \times 10^5$ 1/M·s or faster, binds to human PD-1 with a $k_{assoc}$ of about $9 \times 10^5$ 1/M·s or faster, binds to human PD-1 with a $k_{assoc}$ of about $9.5 \times 10^5$ l/M·s or faster, or binds to human PD-1 with a $k_{assoc}$ of about $1 \times 10^6$ 1/M·s or faster.

**[0006]** In some embodiments, the PD-1 inhibitor is one that binds to human PD-1 with a $k_{dissoc}$ of about $2 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.1 \times 10^{-5}$ 1/s or slower , binds to human PD-1 with a $k_{dissoc}$ of about $2.2 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.3 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.4 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.5 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.6 \times 10^{-5}$ 1/s or slower or binds to human PD-1 with a $k_{dissoc}$ of about $2.7 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.8 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.9 \times 10^{-5}$ 1/s or slower, or binds to human PD-1 with a $k_{dissoc}$ of about $3 \times 10^{-5}$ 1/s or slower.

**[0007]** In some embodiments, the PD-1 inhibitor is one that blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 10 nM or lower, blocks

or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 9 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 8 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 7 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 6 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 5 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 4 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 3 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 2 nM or lower, or blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 1 nM or lower.

**[0008]** In an embodiment, the PD-1 inhibitor is nivolumab (commercially available as OPDIVO from Bristol-Myers Squibb Co.), or biosimilars, antigen-binding fragments, conjugates, or variants thereof. Nivolumab is a fully human IgG4 antibody blocking the PD-1 receptor. In an embodiment, the anti-PD-1 antibody is an immunoglobulin G4 kappa, anti-(human CD274) antibody. Nivolumab is assigned Chemical Abstracts Service (CAS) registry number 946414-94-4 and is also known as 5C4, BMS-936558, MDX-1106, and ONO-4538. The preparation and properties of nivolumab are described in U.S. Patent No. 8,008,449 and International Patent Publication No. WO 2006/121168, the disclosures of which are incorporated by reference herein. The clinical safety and efficacy of nivolumab in various forms of cancer has been described in Wang, *et al.*, *Cancer Immunol Res.* **2014,** 2, 846-56; Page, *et al.*, *Ann. Rev. Med.*, **2014,** *65,* 185-202; and Weber, *et al.*, *J. Clin. Oncology*, **2013,** *31,* 4311-4318, the disclosures of which are incorporated by reference herein. The amino acid sequences of nivolumab are set forth in Table 48. Nivolumab has intra-heavy chain disulfide linkages at 22-96,140-196, 254-314, 360-418, 22"-96", 140"-196", 254"-314", and 360"-418"; intra-light chain disulfide linkages at 23'-88', 134'-194', 23'"-88'", and 134'"-194'"; inter-heavy-light chain disulfide linkages at 127-214', 127"-214'", inter-heavy-heavy chain disulfide linkages at 219-219" and 222-222"; and N-glycosylation sites (H $CH_2$ 84.4) at 290, 290".

**[0009]** In an embodiment, a PD-1 inhibitor comprises a heavy chain given by SEQ ID NO:463 and a light chain given by SEQ ID NO:464. In an embodiment, a PD-1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:463 and SEQ

ID NO:464, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:463 and SEQ ID NO:464, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:463 and SEQ ID NO:464, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:463 and SEQ ID NO:464, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:463 and SEQ ID NO:464, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:463 and SEQ ID NO:464, respectively.

**[0010]** In an embodiment, the PD-1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of nivolumab. In an embodiment, the PD-1 inhibitor heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:465, and the PD-1 inhibitor light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:466, and conservative amino acid substitutions thereof. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:465 and SEQ ID NO:466, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:465 and SEQ ID NO:466, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:465 and SEQ ID NO:466, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:465 and SEQ ID NO:466, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:465 and SEQ ID NO:466, respectively.

**[0011]** In an embodiment, a PD-1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:467, SEQ ID NO:468, and SEQ ID NO:469, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:470, SEQ ID NO:471, and SEQ ID NO:472, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or

binds to the same epitope on PD-1 as any of the aforementioned antibodies.

**[0012]** In an embodiment, the PD-1 inhibitor is an anti-PD-1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to nivolumab. In an embodiment, the biosimilar comprises an anti-PD-1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is nivolumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-1 antibody authorized or submitted for authorization, wherein the anti-PD-1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is nivolumab. The anti-PD-1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is nivolumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is nivolumab.

TABLE 35. Amino acid sequences for PD-1 inhibitors related to nivolumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:463<br>nivolumab<br>heavy chain | QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY<br>ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTLVT VSSASTKGPS<br>VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS<br>VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPPCP PCPAPEFLGG PSVFLFPPKP<br>KDTLMISRTP EVTCVVVDVS QEDPEVQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT<br>VLHQDWLNGK EYKCKVSNKG LPSSIEKTIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLTC<br>LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV<br>MHEALHNHYT QKSLSLSLGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>440 |
| SEQ ID NO:464<br>nivolumab<br>light chain | EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP<br>SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT<br>LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC | 60<br>120<br>180<br>214 |
| SEQ ID NO:465 | QVQLVESGGG VVQPGRSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY | 60 |

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| nivolumab variable heavy chain | ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTLVT VSS | 113 |
| SEQ ID NO:466 nivolumab variable light chain | EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA<br>RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIK | 60<br>107 |
| SEQ ID NO:467 nivolumab heavy chain CDR1 | NSGMH | 5 |
| SEQ ID NO:468 nivolumab heavy chain CDR2 | VIWYDGSKRY YADSVKG | 17 |
| SEQ ID NO:469 nivolumab heavy chain CDR3 | NDDY | 4 |
| SEQ ID NO:470 nivolumab light chain CDR1 | RASQSVSSYL A | 11 |
| SEQ ID NO:471 nivolumab light chain CDR2 | DASNRAT | 7 |
| SEQ ID NO:472 nivolumab light chain CDR3 | QQSSNWPRT | 9 |

**[0013]** In another embodiment, the PD-1 inhibitor comprises pembrolizumab (commercially available as KEYTRUDA from Merck & Co., Inc., Kenilworth, NJ, USA), or antigen-binding fragments, conjugates, or variants thereof. Pembrolizumab is assigned CAS registry number 1374853-91-4 and is also known as lambrolizumab, MK-3475, and SCH-900475. Pembrolizumab has an immunoglobulin G4, anti-(human protein PDCD1 (programmed cell death 1)) (human-Mus musculus monoclonal heavy chain), disulfide with human-Mus musculus monoclonal light chain, dimer structure. The structure of pembrolizumab may also be described as immunoglobulin G4, anti-(human programmed cell death 1); humanized mouse monoclonal [228-L-proline(H10-S>P)]γ4 heavy chain (134-218')-disulfide with humanized mouse monoclonal κ light chain dimer (226-226":229-229")-bisdisulfide. The properties, uses, and preparation of pembrolizumab are described in International Patent Publication No. WO 2008/156712 A1, U.S. Patent No. 8,354,509 and U.S. Patent Application Publication Nos. US 2010/0266617 A1, US 2013/0108651 A1, and US 2013/0109843 A2, the disclosures of which are incorporated herein by reference. The clinical safety and efficacy of pembrolizumab in various forms of cancer is described in Fuerst, *Oncology Times,* **2014,** *36,* 35-36; Robert, *et al.*, *Lancet,* **2014,** *384,* 1109-17; and Thomas, *et al.*, *Exp. Opin. Biol. Ther.*, **2014,** *14,* 1061-1064. The amino acid sequences of

pembrolizumab are set forth in Table 49. Pembrolizumab includes the following disulfide bridges: 22-96, 22"-96", 23'-92', 23'"-92'", 134-218', 134"-218'", 138'-198', 138'"-198'", 147-203, 147"-203", 226-226", 229-229", 261-321, 261"-321", 367-425, and 367"-425", and the following glycosylation sites (N): Asn-297 and Asn-297". Pembrolizumab is an IgG4/kappa isotype with a stabilizing S228P mutation in the Fc region; insertion of this mutation in the IgG4 hinge region prevents the formation of half molecules typically observed for IgG4 antibodies. Pembrolizumab is heterogeneously glycosylated at Asn297 within the Fc domain of each heavy chain, yielding a molecular weight of approximately 149 kDa for the intact antibody. The dominant glycoform of pembrolizumab is the fucosylated agalacto diantennary glycan form (G0F).

**[0014]** In an embodiment, a PD-1 inhibitor comprises a heavy chain given by SEQ ID NO:473 and a light chain given by SEQ ID NO:474. In an embodiment, a PD-1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:473 and SEQ ID NO:474, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:473 and SEQ ID NO:474, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:473 and SEQ ID NO:474, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:473 and SEQ ID NO:474, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:473 and SEQ ID NO:474, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:473 and SEQ ID NO:474, respectively.

**[0015]** In an embodiment, the PD-1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of pembrolizumab. In an embodiment, the PD-1 inhibitor heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:475, and the PD-1 inhibitor light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:476, and conservative amino acid substitutions thereof. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:475 and SEQ ID NO:476, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in

SEQ ID NO:475 and SEQ ID NO:476, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:475 and SEQ ID NO:476, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:475 and SEQ ID NO:476, respectively. In an embodiment, a PD-1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:475 and SEQ ID NO:476, respectively.

**[0016]** In an embodiment, a PD-1 inhibitor comprises the heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:477, SEQ ID NO:478, and SEQ ID NO:479, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:480, SEQ ID NO:481, and SEQ ID NO:482, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-1 as any of the aforementioned antibodies.

**[0017]** In an embodiment, the PD-1 inhibitor is an anti-PD-1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to pembrolizumab. In an embodiment, the biosimilar comprises an anti-PD-1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is pembrolizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-1 antibody authorized or submitted for authorization, wherein the anti-PD-1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is pembrolizumab. The anti-PD-1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is pembrolizumab.

In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is pembrolizumab.

TABLE 36. Amino acid sequences for PD-1 inhibitors related to pembrolizumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:473 pembrolizumab heavy chain | QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF<br>NEKFKNRVTL TTDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS<br>ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS<br>GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPPCP APEFLGGPSV<br>FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY<br>RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK<br>NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSRL TVDKSRWQEG<br>NVFSCSVMHE ALHNHYTQKS LSLSLGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>447 |
| SEQ ID NO:474 pembrolizumab light chain | EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES<br>GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI KRTVAAPSVF<br>IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSLS<br>STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC | 60<br>120<br>180<br>218 |
| SEQ ID NO:475 pembrolizumab variable heavy chain | QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF<br>NEKFKNRVTL TTDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS | 60<br>120 |
| SEQ ID NO:476 pembrolizumab variable light chain | EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES<br>GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI K | 60<br>111 |
| SEQ ID NO:477 pembrolizumab heavy chain CDR1 | NYYMY | 5 |
| SEQ ID NO:478 pembrolizumab heavy chain CDR2 | GINPSNGGTN FNEKFK | 16 |
| SEQ ID NO:479 pembrolizumab heavy chain CDR3 | RDYRFDMGFD Y | 11 |
| SEQ ID NO:480 pembrolizumab light chain CDR1 | RASKGVSTSG YSYLH | 15 |
| SEQ ID NO:481 pembrolizumab light chain CDR2 | LASYLES | 7 |
| SEQ ID NO:482 pembrolizumab light chain CDR3 | QHSRDLPLT | 9 |

**[0018]** In an embodiment, the PD-1 inhibitor is a commercially-available anti-PD-1 monoclonal antibody, such as anti-m-PD-1 clones J43 (Cat # BE0033-2) and RMP1-14 (Cat # BE0146) (Bio X Cell, Inc., West Lebanon, NH, USA). A number of commercially-available anti-PD-1 antibodies are known to one of ordinary skill in the art.

**[0019]** In an embodiment, the PD-1 inhibitor is an antibody disclosed in U.S. Patent No. 8,354,509 or U.S. Patent Application Publication Nos. 2010/0266617 A1, 2013/0108651 A1,

2013/0109843 A2, the disclosures of which are incorporated by reference herein. In an embodiment, the PD-1 inhibitor is an anti-PD-1 antibody described in U.S. Patent Nos. 8,287,856, 8,580,247, and 8,168,757 and U.S. Patent Application Publication Nos. 2009/0028857 A1, 2010/0285013 A1, 2013/0022600 A1, and 2011/0008369 A1, the teachings of which are hereby incorporated by reference. In another embodiment, the PD-1 inhibitor is an anti-PD-1 antibody disclosed in U.S. Patent No. 8,735,553 B1, the disclosure of which is incorporated herein by reference. In an embodiment, the PD-1 inhibitor is pidilizumab, also known as CT-011, which is described in U.S. Patent No. 8,686,119, the disclosure of which is incorporated by reference herein.

**[0020]** In an embodiment, the PD-1 inhibitor may be a small molecule or a peptide, or a peptide derivative, such as those described in U.S. Patent Nos. 8,907,053; 9,096,642; and 9,044,442 and U.S. Patent Application Publication No. US 2015/0087581; 1,2,4-oxadiazole compounds and derivatives such as those described in U.S. Patent Application Publication No. 2015/0073024; cyclic peptidomimetic compounds and derivatives such as those described in U.S. Patent Application Publication No. US 2015/0073042; cyclic compounds and derivatives such as those described in U.S. Patent Application Publication No. US 2015/0125491; 1,3,4-oxadiazole and 1,3,4-thiadiazole compounds and derivatives such as those described in International Patent Application Publication No. WO 2015/033301; peptide-based compounds and derivatives such as those described in International Patent Application Publication Nos. WO 2015/036927 and WO 2015/04490, or a macrocyclic peptide-based compounds and derivatives such as those described in U.S. Patent Application Publication No. US 2014/0294898; the disclosures of each of which are hereby incorporated by reference in their entireties.

**[0021]** In an embodiment, the PD-L1 or PD-L2 inhibitor may be any PD-L1 or PD-L2 inhibitor, antagonist, or blocker known in the art. In particular, it is one of the PD-L1 or PD-L2 inhibitors, antagonist, or blockers described in more detail in the following paragraphs. The terms "inhibitor," "antagonist," and "blocker" are used interchangeably herein in reference to PD-L1 and PD-L2 inhibitors. For avoidance of doubt, references herein to a PD-L1 or PD-L2 inhibitor that is an antibody may refer to a compound or antigen-binding fragments, variants, conjugates, or biosimilars thereof. For avoidance of doubt, references herein to a PD-L1 or PD-L2 inhibitor may refer to a compound or a pharmaceutically acceptable salt, ester, solvate, hydrate, cocrystal, or prodrug thereof.

**[0022]** In some embodiments, the compositions, processes and methods described herein include a PD-L1 or PD-L2 inhibitor. In some embodiments, the PD-L1 or PD-L2 inhibitor is a small molecule. In a preferred embodiment, the PD-L1 or PD-L2 inhibitor is an antibody (*i.e.*, an anti-PD-1 antibody), a fragment thereof, including Fab fragments, or a single-chain variable fragment (scFv) thereof. In some embodiments the PD-L1 or PD-L2 inhibitor is a polyclonal antibody. In a preferred embodiment, the PD-L1 or PD-L2 inhibitor is a monoclonal antibody. In some embodiments, the PD-L1 or PD-L2 inhibitor competes for binding with PD-L1 or PD-L2, and/or binds to an epitope on PD-L1 or PD-L2. In an embodiment, the antibody competes for binding with PD-L1 or PD-L2, and/or binds to an epitope on PD-L1 or PD-L2.

**[0023]** In some embodiments, the PD-L1 inhibitors provided herein are selective for PD-L1, in that the compounds bind or interact with PD-L1 at substantially lower concentrations than they bind or interact with other receptors, including the PD-L2 receptor. In certain embodiments, the compounds bind to the PD-L1 receptor at a binding constant that is at least about a 2-fold higher concentration, about a 3-fold higher concentration, about a 5-fold higher concentration, about a 10-fold higher concentration, about a 20-fold higher concentration, about a 30-fold higher concentration, about a 50-fold higher concentration, about a 100-fold higher concentration, about a 200-fold higher concentration, about a 300-fold higher concentration, or about a 500-fold higher concentration than to the PD-L2 receptor.

**[0024]** In some embodiments, the PD-L2 inhibitors provided herein are selective for PD-L2, in that the compounds bind or interact with PD-L2 at substantially lower concentrations than they bind or interact with other receptors, including the PD-L1 receptor. In certain embodiments, the compounds bind to the PD-L2 receptor at a binding constant that is at least about a 2-fold higher concentration, about a 3-fold higher concentration, about a 5-fold higher concentration, about a 10-fold higher concentration, about a 20-fold higher concentration, about a 30-fold higher concentration, about a 50-fold higher concentration, about a 100-fold higher concentration, about a 200-fold higher concentration, about a 300-fold higher concentration, or about a 500-fold higher concentration than to the PD-L1 receptor.

**[0025]** Without being bound by any theory, it is believed that tumor cells express PD-L1, and that T cells express PD-1. However, PD-L1 expression by tumor cells is not required for efficacy of PD-1 or PD-L1 inhibitors or blockers. In an embodiment, the tumor cells express

PD-L1. In another embodiment, the tumor cells do not express PD-L1. In some embodiments, the methods can include a combination of a PD-1 and a PD-L1 antibody, such as those described herein, in combination with a TIL. The administration of a combination of a PD-1 and a PD-L1 antibody and a TIL may be simultaneous or sequential.

**[0026]** In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that binds human PD-L1 and/or PD-L2 with a $K_D$ of about 100 pM or lower, binds human PD-L1 and/or PD-L2 with a $K_D$ of about 90 pM or lower, binds human PD-L1 and/or PD-L2 with a $K_D$ of about 80 pM or lower, binds human PD-L1 and/or PD-L2 with a $K_D$ of about 70 pM or lower, binds human PD-L1 and/or PD-L2 with a $K_D$ of about 60 pM or lower, a $K_D$ of about 50 pM or lower, binds human PD-L1 and/or PD-L2 with a $K_D$ of about 40 pM or lower, or binds human PD-L1 and/or PD-L2 with a KD of about 30 pM or lower,

**[0027]** In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that binds to human PD-L1 and/or PD-L2 with a $k_{assoc}$ of about $7.5 \times 10^5$ 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a $k_{assoc}$ of about $8 \times 10^5$ 1/M·s or faster, binds to human PD-L1 and/ or PD-L2 with a $k_{assoc}$ of about $8.5 \times 10^5$ 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a $k_{assoc}$ of about $9 \times 10^5$ 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a $k_{assoc}$ of about $9.5 \times 10^5$ 1/M·s and/or faster, or binds to human PD-L1 and/or PD-L2 with a $k_{assoc}$ of about $1 \times 10^6$ 1/M·s or faster.

**[0028]** In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that binds to human PD-L1 or PD-L2 with a $k_{dissoc}$ of about $2 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.1 \times 10^{-5}$ 1/s or slower , binds to human PD-1 with a $k_{dissoc}$ of about $2.2 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.3 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.4 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.5 \times 10^{-5}$ 1/s or slower, binds to human PD-1 with a $k_{dissoc}$ of about $2.6 \times 10^{-5}$ 1/s or slower, binds to human PD-L1 or PD-L2 with a $k_{dissoc}$ of about $2.7 \times 10^{-5}$ 1/s or slower, or binds to human PD-L1 or PD-L2 with a $k_{dissoc}$ of about $3 \times 10^{-5}$ 1/s or slower.

**[0029]** In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 10 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 9 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 8 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 7 nM or lower; blocks

or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 6 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 5 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 4 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 3 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an $IC_{50}$ of about 2 nM or lower; or blocks human PD-1, or blocks binding of human PD-L1 or human PD-L2 to human PD-l with an $IC_{50}$ of about 1 nM or lower.

**[0030]** In an embodiment, the PD-L1 inhibitor is durvalumab, also known as MEDI4736 (which is commercially available from Medimmune, LLC, Gaithersburg, Maryland, a subsidiary of AstraZeneca plc.), or antigen-binding fragments, conjugates, or variants thereof. In an embodiment, the PD-L1 inhibitor is an antibody disclosed in U.S. Patent No. 8,779,108 or U.S. Patent Application Publication No. 2013/0034559, the disclosures of which are incorporated by reference herein. The clinical efficacy of durvalumab has been described in Page, *et al.*, *Ann. Rev. Med.*, **2014,** *65,* 185-202; Brahmer, *et al.*, *J. Clin. Oncol.* **2014,** *32,* 5s (supplement, abstract 8021); and McDermott, *et al.*, *Cancer Treatment Rev.*, **2014,** *40,* 1056-64. The preparation and properties of durvalumab are described in U.S. Patent No. 8,779,108, the disclosure of which is incorporated by reference herein. The amino acid sequences of durvalumab are set forth in Table 50. The durvalumab monoclonal antibody includes disulfide linkages at 22-96, 22"-96", 23'-89', 23'"-89'", 135'-195', 135'"-195'", 148-204, 148"-204", 215'-224, 215'"-224", 230-230", 233-233", 265-325, 265"-325", 371-429, and 371"-429'; and N-glycosylation sites at Asn-301 and Asn-301".

**[0031]** In an embodiment, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:483 and a light chain given by SEQ ID NO:484. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:483 and SEQ ID NO:484, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:483 and SEQ ID NO:484, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:483 and SEQ ID NO:484, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:483 and SEQ ID NO:484, respectively. In an embodiment, a PD-L1 inhibitor

comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:483 and SEQ ID NO:484, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:483 and SEQ ID NO:484, respectively.

**[0032]** In an embodiment, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of durvalumab. In an embodiment, the PD-L1 inhibitor heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:485, and the PD-L1 inhibitor light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:486, and conservative amino acid substitutions thereof. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:485 and SEQ ID NO:486, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:485 and SEQ ID NO:486, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:485 and SEQ ID NO:486, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:485 and SEQ ID NO:486, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:485 and SEQ ID NO:486, respectively.

**[0033]** In an embodiment, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:487, SEQ ID NO:488, and SEQ ID NO:489, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:490, SEQ ID NO:491, and SEQ ID NO:492, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

**[0034]** In an embodiment, the PD-L1 inhibitor is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to durvalumab. In an embodiment, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal

product or reference biological product is durvalumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab.

TABLE 37. Amino acid sequences for PD-L1 inhibitors related to durvalumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:483 durvalumab heavy chain | EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN IKQDGSEKYY<br>VDSVKGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCAREG GWFGELAFDY WGQGTLVTVS<br>SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS<br>SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPEFEG<br>GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY<br>NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPASIEKTI SKAKGQPREP QVYTLPPSRE<br>EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR<br>WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>451 |
| SEQ ID NO:484 durvalumab light chain | EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN EIVLTQSPGT<br>LSLSPGERAT LSCRASQRVS SSYLAWYQQK PGQAPRLLIY DASSRATGIP DRFSGSGSGT<br>DFTLTISRLE PEDFAVYYCQ QYGSLPWTFG QGTKVEIKRT VAAPSVFIFP PSDEQLKSGT<br>ASVVCLLNNF YPREAKVQWK VDNALQSGNS QESVTEQDSK DSTYSLSSTL TLSKADYEKH<br>KVYACEVTHQ GLSSPVTKSF NRGEC | 60<br>120<br>180<br>240<br>265 |
| SEQ ID NO:485 durvalumab variable heavy chain | EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN IKQDGSEKYY<br>VDSVKGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCAREG GWFGELAFDY WGQGTLVTVS<br>S | 60<br>120<br>121 |
| SEQ ID NO:486 durvalumab variable light chain | EIVLTQSPGT LSLSPGERAT LSCRASQRVS SSYLAWYQQK PGQAPRLLIY DASSRATGIP<br>DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSLPWTFG QGTKVEIK | 60<br>108 |
| SEQ ID NO:487 durvalumab heavy chain CDR1 | RYWMS | 5 |
| SEQ ID NO:488 durvalumab heavy chain CDR2 | NIKQDGSEKY YVDSVKG | 17 |
| SEQ ID NO:489 durvalumab | EGGWFGELAF DY | 12 |

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| heavy chain CDR3 | | |
| SEQ ID NO:490 durvalumab light chain CDR1 | RASQRVSSSY LA | 12 |
| SEQ ID NO:491 durvalumab light chain CDR2 | DASSRAT | 7 |
| SEQ ID NO:492 durvalumab light chain CDR3 | QQYGSLPWT | 9 |

**[0035]** In an embodiment, the PD-L1 inhibitor is avelumab, also known as MSB0010718C (commercially available from Merck KGaA/EMD Serono), or antigen-binding fragments, conjugates, or variants thereof. The preparation and properties of avelumab are described in U.S. Patent Application Publication No. US 2014/0341917 A1, the disclosure of which is specifically incorporated by reference herein. The amino acid sequences of avelumab are set forth in Table 51. Avelumab has intra-heavy chain disulfide linkages (C23-C104) at 22-96, 147-203, 264-324, 370-428, 22"-96", 147"-203", 264"-324", and 370"-428"; intra-light chain disulfide linkages (C23-C104) at 22'-90', 138'-197', 22'"-90'", and 138'"-197'"; intra-heavy-light chain disulfide linkages (h 5-CL 126) at 223-215' and 223"-215'"; intra-heavy-heavy chain disulfide linkages (h 11, h 14) at 229-229" and 232-232"; N-glycosylation sites (H $CH_2$ N84.4) at 300, 300"; fucosylated complex bi-antennary CHO-type glycans; and H CHS K2 C-terminal lysine clipping at 450 and 450'.

**[0036]** In an embodiment, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:493 and a light chain given by SEQ ID NO:494. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:493 and SEQ ID NO:494, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:493 and SEQ ID NO:494, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:493 and SEQ ID NO:494, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:493 and SEQ ID NO:494, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown

in SEQ ID NO:493 and SEQ ID NO:494, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:493 and SEQ ID NO:494, respectively.

**[0037]** In an embodiment, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of avelumab. In an embodiment, the PD-L1 inhibitor heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:495, and the PD-L1 inhibitor light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:496, and conservative amino acid substitutions thereof. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:495 and SEQ ID NO:496, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:496 and SEQ ID NO:496, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:495 and SEQ ID NO:496, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:495 and SEQ ID NO:496, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:495 and SEQ ID NO:496, respectively.

**[0038]** In an embodiment, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:497, SEQ ID NO:498, and SEQ ID NO:499, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:500, SEQ ID NO:501, and SEQ ID NO:502, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

**[0039]** In an embodiment, the PD-L1 inhibitor is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to avelumab. In an embodiment, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. In some embodiments, the one or more

post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab.

TABLE 38. Amino acid sequences for PD-L1 inhibitors related to avelumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:493 avelumab heavy chain | EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS IYPSGGITFY<br>ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK LGTVTTVDYW GQGTLVTVSS<br>ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS<br>GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG<br>PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN<br>STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE<br>LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW<br>QQGNVFSCSV MHEALHNHYT QKSLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>450 |
| SEQ ID NO:494 avelumab light chain | QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLMI YDVSNRPSGV<br>SNRFSGSKSG NTASLTISGL QAEDEADYYC SSYTSSSTRV FGTGTKVTVL GQPKANPTVT<br>LFPPSSEELQ ANKATLVCLI SDFYPGAVTV AWKADGSPVK AGVETTKPSK QSNNKYAASS<br>YLSLTPEQWK SHRSYSCQVT HEGSTVEKTV APTECS | 60<br>120<br>180<br>216 |
| SEQ ID NO:495 avelumab variable heavy chain | EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS IYPSGGITFY<br>ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK LGTVTTVDYW GQGTLVTVSS | 60<br>120 |
| SEQ ID NO:496 avelumab variable light chain | QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLMI YDVSNRPSGV<br>SNRFSGSKSG NTASLTISGL QAEDEADYYC SSYTSSSTRV FGTGTKVTVL | 60<br>110 |
| SEQ ID NO:497 avelumab heavy chain CDR1 | SYIMM | 5 |
| SEQ ID NO:498 avelumab heavy chain CDR2 | SIYPSGGITF YADTVKG | 17 |
| SEQ ID NO:499 avelumab heavy chain CDR3 | IKLGTVTTVD Y | 11 |
| SEQ ID NO:500 avelumab | TGTSSDVGGY NYVS | 14 |

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| light chain CDR1 | | |
| SEQ ID NO:501 avelumab light chain CDR2 | DVSNRPS | 7 |
| SEQ ID NO:502 avelumab light chain CDR3 | SSYTSSSTRV | 10 |

**[0040]** In an embodiment, the PD-L1 inhibitor is atezolizumab, also known as MPDL3280A or RG7446 (commercially available as TECENTRIQ from Genentech, Inc., a subsidiary of Roche Holding AG, Basel, Switzerland), or antigen-binding fragments, conjugates, or variants thereof. In an embodiment, the PD-L1 inhibitor is an antibody disclosed in U.S. Patent No. 8,217,149, the disclosure of which is specifically incorporated by reference herein. In an embodiment, the PD-L1 inhibitor is an antibody disclosed in U.S. Patent Application Publication Nos. 2010/0203056 A1, 2013/0045200 A1, 2013/0045201 A1, 2013/0045202 A1, or 2014/0065135 A1, the disclosures of which are specifically incorporated by reference herein. The preparation and properties of atezolizumab are described in U.S. Patent No. 8,217,149, the disclosure of which is incorporated by reference herein. The amino acid sequences of atezolizumab are set forth in Table 52. Atezolizumab has intra-heavy chain disulfide linkages (C23-C104) at 22-96, 145-201, 262-322, 368-426, 22"-96", 145"-201", 262"-322", and 368"-426"; intra-light chain disulfide linkages (C23-C104) at 23'-88', 134'-194', 23'"-88'", and 134'"-194'"; intra-heavy-light chain disulfide linkages (h 5-CL 126) at 221-214' and 221"-214'"; intra-heavy-heavy chain disulfide linkages (h 11, h 14) at 227-227" and 230-230"; and N-glycosylation sites (H $CH_2$ N84.4>A) at 298 and 298'.

**[0041]** In an embodiment, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:503 and a light chain given by SEQ ID NO:504. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:503 and SEQ ID NO:504, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:503 and SEQ ID NO:504, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:503 and SEQ ID NO:504, respectively. In an embodiment, a PD-L1 inhibitor

comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:503 and SEQ ID NO:504, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:503 and SEQ ID NO:504, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:503 and SEQ ID NO:504, respectively.

**[0042]** In an embodiment, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of atezolizumab. In an embodiment, the PD-L1 inhibitor heavy chain variable region ($V_H$) comprises the sequence shown in SEQ ID NO:505, and the PD-L1 inhibitor light chain variable region ($V_L$) comprises the sequence shown in SEQ ID NO:506, and conservative amino acid substitutions thereof. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 99% identical to the sequences shown in SEQ ID NO:505 and SEQ ID NO:506, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 98% identical to the sequences shown in SEQ ID NO:505 and SEQ ID NO:506, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 97% identical to the sequences shown in SEQ ID NO:505 and SEQ ID NO:506, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 96% identical to the sequences shown in SEQ ID NO:505 and SEQ ID NO:506, respectively. In an embodiment, a PD-L1 inhibitor comprises $V_H$ and $V_L$ regions that are each at least 95% identical to the sequences shown in SEQ ID NO:505 and SEQ ID NO:506, respectively.

**[0043]** In an embodiment, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:507, SEQ ID NO:508, and SEQ ID NO:509, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:510, SEQ ID NO:511, and SEQ ID NO:512, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

**[0044]** In an embodiment, the anti-PD-L1 antibody is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to atezolizumab. In an embodiment, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference

biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is atezolizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is atezolizumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is atezolizumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is atezolizumab.

TABLE 39. Amino acid sequences for PD-L1 inhibitors related to atezolizumab.

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| SEQ ID NO:503 atezolizumab heavy chain | EVQLVESGGG LVQPGGSLRL SCAASGFTFS DSWIHWVRQA PGKGLEWVAW ISPYGGSTYY<br>ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCARRH WPGGFDYWGQ GTLVTVSSAS<br>TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL<br>YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTCPPC PAPELLGGPS<br>VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYAST<br>YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT<br>KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSK LTVDKSRWQQ<br>GNVFSCSVMH EALHNHYTQK SLSLSPGK | 60<br>120<br>180<br>240<br>300<br>360<br>420<br>448 |
| SEQ ID NO:504 atezolizumab light chain | DIQMTQSPSS LSASVGDRVT ITCRASQDVS TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS<br>RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YLYHPATFGQ GTKVEIKRTV AAPSVFIFPP<br>SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT<br>LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC | 60<br>120<br>180<br>214 |
| SEQ ID NO:505 atezolizumab variable heavy chain | EVQLVESGGG LVQPGGSLRL SCAASGFTFS DSWIHWVRQA PGKGLEWVAW ISPYGGSTYY<br>ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCARRH WPGGFDYWGQ GTLVTVSA | 60<br>118 |
| SEQ ID NO:506 atezolizumab variable light chain | DIQMTQSPSS LSASVGDRVT ITCRASQDVS TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS<br>RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YLYHPATFGQ GTKVEIKR | 60<br>108 |
| SEQ ID NO:507 atezolizumab heavy chain CDR1 | GFTFSDSWIH | 10 |
| SEQ ID NO:508 | AWISPYGGST YYADSVKG | 18 |

| Identifier | Sequence (One-Letter Amino Acid Symbols) | |
|---|---|---|
| atezolizumab heavy chain CDR2 | | |
| SEQ ID NO:509 atezolizumab heavy chain CDR3 | RHWPGGFDY | 9 |
| SEQ ID NO:510 atezolizumab light chain CDR1 | RASQDVSTAV A | 11 |
| SEQ ID NO:511 atezolizumab light chain CDR2 | SASFLYS | 7 |
| SEQ ID NO:512 atezolizumab light chain CDR3 | QQYLYHPAT | 9 |

**[0045]** In an embodiment, PD-L1 inhibitors include those antibodies described in U.S. Patent Application Publication No. US 2014/0341917 A1, the disclosure of which is incorporated by reference herein. In another embodiment, antibodies that compete with any of these antibodies for binding to PD-L1 are also included. In an embodiment, the anti-PD-L1 antibody is MDX-1105, also known as BMS-935559, which is disclosed in U.S. Patent No. US 7,943,743, the disclosures of which are incorporated by reference herein. In an embodiment, the anti-PD-L1 antibody is selected from the anti-PD-L1 antibodies disclosed in U.S. Patent No. US 7,943,743, which are incorporated by reference herein.

**[0046]** In an embodiment, the PD-L1 inhibitor is a commercially-available monoclonal antibody, such as INVIVOMAB anti-m-PD-L1 clone 10F.9G2 (Catalog # BE0101, Bio X Cell, Inc., West Lebanon, NH, USA). In an embodiment, the anti-PD-L1 antibody is a commercially-available monoclonal antibody, such as AFFYMETRIX EBIOSCIENCE (MIH1). A number of commercially-available anti-PD-L1 antibodies are known to one of ordinary skill in the art.

**[0047]** In an embodiment, the PD-L2 inhibitor is a commercially-available monoclonal antibody, such as BIOLEGEND 24F.10C12 Mouse IgG2a, κ isotype (catalog # 329602 Biolegend, Inc., San Diego, CA), SIGMA anti-PD-L2 antibody (catalog # SAB3500395, Sigma-Aldrich Co., St. Louis, MO), or other commercially-available anti-PD-L2 antibodies known to one of ordinary skill in the art.

## 2. Optional Lymphodepletion Preconditioning of Patients

**[001533]** In an embodiment, the invention includes a method of treating a cancer with a population of TILs, wherein a patient is pre-treated with non-myeloablative chemotherapy prior to an infusion of TILs according to the present disclosure. In an embodiment, the invention includes a population of TILs for use in the treatment of cancer in a patient which has been pre-treated with non-myeloablative chemotherapy. In an embodiment, the population of TILs is for administration by infusion. In an embodiment, the non-myeloablative chemotherapy is cyclophosphamide 60 mg/kg/d for 2 days (days 27 and 26 prior to TIL infusion) and fludarabine 25 mg/m$^2$/d for 5 days (days 27 to 23 prior to TIL infusion). In an embodiment, after non-myeloablative chemotherapy and TIL infusion (at day 0) according to the present disclosure, the patient receives an intravenous infusion of IL-2 (aldesleukin, commercially available as PROLEUKIN) intravenously at 720,000 IU/kg every 8 hours to physiologic tolerance. In certain embodiments, the population of TILs is for use in treating cancer in combination with IL-2, wherein the IL-2 is administered after the population of TILs.

**[001534]** Experimental findings indicate that lymphodepletion prior to adoptive transfer of tumor-specific T lymphocytes plays a key role in enhancing treatment efficacy by eliminating regulatory T cells and competing elements of the immune system ('cytokine sinks'). Accordingly, some embodiments of the invention utilize a lymphodepletion step (sometimes also referred to as "immunosuppressive conditioning") on the patient prior to the introduction of the TILs of the invention.

**[001535]** In general, lymphodepletion is achieved using administration of fludarabine or cyclophosphamide (the active form being referred to as mafosfamide) and combinations thereof. Such methods are described in Gassner, *et al., Cancer Immunol. Immunother*. **2011**, *60,* 75–85, Muranski, *et al.*, *Nat. Clin. Pract. Oncol.,* **2006,** *3,* 668–681, Dudley, *et al.*, *J. Clin. Oncol.* **2008**, *26,* 5233-5239, and Dudley, *et al.*, *J. Clin. Oncol.* **2005**, *23,* 2346–2357, all of which are incorporated by reference herein in their entireties.

**[001536]** In some embodiments, the fludarabine is administered at a concentration of 0.5 μg/mL -10 μg/mL fludarabine. In some embodiments, the fludarabine is administered at a concentration of 1 μg/mL fludarabine. In some embodiments, the fludarabine treatment is administered for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days or more. In some embodiments, the fludarabine is administered at a dosage of 10 mg/kg/day, 15 mg/kg/day, 20 mg/kg/day, 25 mg/kg/day, 30 mg/kg/day, 35 mg/kg/day, 40 mg/kg/day, or 45 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 2-7 days at

35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4-5 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4-5 days at 25 mg/kg/day.

**[001537]** In some embodiments, the mafosfamide, the active form of cyclophosphamide, is obtained at a concentration of 0.5 μg/mL -10 μg/mL by administration of cyclophosphamide. In some embodiments, mafosfamide, the active form of cyclophosphamide, is obtained at a concentration of 1 μg/mL by administration of cyclophosphamide. In some embodiments, the cyclophosphamide treatment is administered for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days or more. In some embodiments, the cyclophosphamide is administered at a dosage of 100 mg/m$^2$/day, 150 mg/m$^2$/day, 175 mg/m$^2$/day, 200 mg/m$^2$/day, 225 mg/m$^2$/day, 250 mg/m$^2$/day, 275 mg/m$^2$/day, or 300 mg/m$^2$/day. In some embodiments, the cyclophosphamide is administered intravenously *(i.e.*, i.v.) In some embodiments, the cyclophosphamide treatment is administered for 2-7 days at 35 mg/kg/day. In some embodiments, the cyclophosphamide treatment is administered for 4-5 days at 250 mg/m$^2$/day i.v. In some embodiments, the cyclophosphamide treatment is administered for 4 days at 250 mg/m$^2$/day i.v.

**[001538]** In some embodiments, lymphodepletion is performed by administering the fludarabine and the cyclophosphamide together to a patient. In some embodiments, fludarabine is administered at 25 mg/m$^2$/day i.v. and cyclophosphamide is administered at 250 mg/m$^2$/day i.v. over 4 days.

**[001539]** In an embodiment, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

### 3. IL-2 Regimens

**[001540]** In an embodiment, the IL-2 regimen comprises a high-dose IL-2 regimen, wherein the high-dose IL-2 regimen comprises aldesleukin, or a biosimilar or variant thereof, administered intravenously starting on the day after administering a therapeutically effective portion of therapeutic population of TILs, wherein the aldesleukin or a biosimilar or variant thereof is administered at a dose of 0.037 mg/kg or 0.044 mg/kg IU/kg (patient body mass) using 15-minute bolus intravenous infusions every eight hours until tolerance, for a maximum of 14 doses. Following 9 days of rest, this schedule may be repeated for another 14 doses, for a maximum of 28 doses in total. In some embodiments, IL-2 is administered in 1,

2, 3, 4, 5, or 6 doses. In some embodiemtns, IL-2 is administered at a maximum dosage of up to 6 doses.

**[001541]** In an embodiment, the IL-2 regimen comprises a decrescendo IL-2 regimen. Decrescendo IL-2 regimens have been described in O'Day, *et al.*, *J. Clin. Oncol.* **1999,** *17,* 2752-61 and Eton, *et al.*, *Cancer* **2000,** *88,* 1703-9, the disclosures of which are incorporated herein by reference. In an embodiment, a decrescendo IL-2 regimen comprises $18 \times 10^6$ IU/m$^2$ administered intravenously over 6 hours, followed by $18 \times 10^6$ IU/m$^2$ administered intravenously over 12 hours, followed by $18 \times 10^6$ IU/m$^2$ administered intravenously over 24 hrs, followed by $4.5 \times 10^6$ IU/m$^2$ administered intravenously over 72 hours. This treatment cycle may be repeated every 28 days for a maximum of four cycles. In an embodiment, a decrescendo IL-2 regimen comprises 18,000,000 IU/m$^2$ on day 1, 9,000,000 IU/m$^2$ on day 2, and 4,500,000 IU/m$^2$ on days 3 and 4.

**[001542]** In an embodiment, the IL-2 regimen comprises administration of pegylated IL-2 every 1, 2, 4, 6, 7, 14 or 21 days at a dose of 0.10 mg/day to 50 mg/day.

### 4. Additional Methods of Treatment

**[001543]** In another embodiment, the invention provides a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the therapeutic TIL population described in any of the preceding paragraphs above.

**[001544]** In another embodiment, the invention provides a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the TIL composition described in any of the preceding paragraphs above.

**[001545]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that prior to administering the therapeutically effective dosage of the therapeutic TIL population and the TIL composition described in any of the preceding paragraphs above, respectively, a non-myeloablative lymphodepletion regimen has been administered to the subject.

**[001546]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the non-myeloablative lymphodepletion regimen comprises the steps of administration of

cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

**[001547]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified to further comprise the step of treating the subject with a high-dose IL-2 regimen starting on the day after administration of the TIL cells to the subject.

**[001548]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the high-dose IL-2 regimen comprises 600,000 or 720,000 IU/kg administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

**[001549]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is a solid tumor.

**[001550]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, or renal cell carcinoma.

**[001551]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is melanoma, HNSCC, cervical cancers, NSCLC, glioblastoma (including GBM), and gastrointestinal cancer.

**[001552]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above e modified such that the cancer is melanoma.

**[001553]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is HNSCC.

**[001554]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is a cervical cancer.

**[001555]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is NSCLC.

**[001556]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is glioblastoma (including GBM).

**[001557]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is gastrointestinal cancer.

**[001558]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is a hypermutated cancer.

**[001559]** In another embodiment, the invention provides the method for treating a subject with cancer described in any of the preceding paragraphs above modified such that the cancer is a pediatric hypermutated cancer.

**[001560]** In another embodiment, the invention provides the therapeutic TIL population described in any of the preceding paragraphs above for use in a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the therapeutic TIL population.

**[001561]** In another embodiment, the invention provides the TIL composition described in any of the preceding paragraphs above for use in a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the TIL composition.

**[001562]** In another embodiment, the invention provides the therapeutic TIL population described in any of the preceding paragraphs above or the TIL composition described in any of the preceding paragraphs above modified such that prior to administering to the subject the therapeutically effective dosage of the therapeutic TIL population described in any of the preceding paragraphs above or the TIL composition described in any of the preceding

paragraphs above, a non-myeloablative lymphodepletion regimen has been administered to the subject.

**[001563]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

**[001564]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified to further comprise the step of treating patient with a high-dose IL-2 regimen starting on the day after administration of the TIL cells to the patient.

**[001565]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the high-dose IL-2 regimen comprises 600,000 or 720,000 IU/kg administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

**[001566]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is a solid tumor.

**[001567]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, or renal cell carcinoma.

**[001568]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is melanoma, HNSCC, cervical cancers, NSCLC, glioblastoma (including GBM), and gastrointestinal cancer.

**[001569]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is melanoma.

**[001570]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is HNSCC.

**[001571]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is a cervical cancer.

**[001572]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is NSCLC.

**[001573]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is glioblastoma (including GBM).

**[001574]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is gastrointestinal cancer.

**[001575]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is a hypermutated cancer.

**[001576]** In another embodiment, the invention provides the therapeutic TIL population or the TIL composition described in any of the preceding paragraphs above modified such that the cancer is a pediatric hypermutated cancer.

**[001577]** In another embodiment, the invention provides the use of the therapeutic TIL population described in any of any of the preceding paragraphs above in a method of treating cancer in a subject comprising administering to the subject a therapeutically effective dosage of the therapeutic TIL population.

**[001578]** In another embodiment, the invention provides the use of the TIL composition described in any of the preceding paragraphs above in a method of treating cancer in a subject comprising administering to the subject a therapeutically effective dosage of the TIL composition.

**[001579]** In another embodiment, the invention provides the use of the therapeutic TIL population described in any of the preceding paragraphs above or the TIL composition

described in any of the preceding paragraphs above in a method of treating cancer in a subject comprising administering to the subject a non-myeloablative lymphodepletion regimen and then administering to the subject the therapeutically effective dosage of the therapeutic TIL population described in any of the preceding paragraphs above or the therapeutically effective dosage of the TIL composition described in any of the preceding paragraphs above.

## EXAMPLES

**[001580]** The embodiments encompassed herein are now described with reference to the following examples. These examples are provided for the purpose of illustration only and the disclosure encompassed herein should in no way be construed as being limited to these examples, but rather should be construed to encompass any and all variations which become evident as a result of the teachings provided herein.

### EXAMPLE 1: PREPARATION OF MEDIA FOR PRE-REP AND REP PROCESSES

**[001581]** This Example describes the procedure for the preparation of tissue culture media for use in protocols involving the culture of tumor infiltrating lymphocytes (TIL) derived from various tumor types including non-small cell lung carcinoma (NSCLC). This media can be used for preparation of any of the TILs described in the present application and Examples.

**Preparation of CM1**

**[001582]** Removed the following reagents from cold storage and warmed them in a 37°C water bath: (RPMI1640, Human AB serum, 200mM L-glutamine). Prepared CM1 medium according to Table 18 below by adding each of the ingredients into the top section of a 0.2um filter unit appropriate to the volume to be filtered. Store at 4°C.

**TABLE 40: Preparation of CM1**

| Ingredient | Final concentration | Final Volume 500 ml | Final Volume IL |
|---|---|---|---|
| RPMI1640 | NA | 450 ml | 900 m1 |
| Human AB serum, heat-inactivated 10% | 50 ml | 100 ml | |
| 200mM L-glutamine | 2 mM | 5 ml | 10 m1 |
| 55mM BME | 55 µM | 0.5 ml | 1 ml |
| 50mg/ml gentamicin sulfate | 50 µg/ml | 0.5 ml | 1 ml |

**[001583]** On the day of use, prewarmed required amount of CM1 in 37°C water bath and add 6000 IU/m1 IL-2.

**[001584]** Additional supplementation - as needed according to Table 41.

**TABLE 41: Additional supplementation of CM1, as needed.**

| Supplement | Stock concentration | Dilution | Final concentration |
|---|---|---|---|
| GlutaMAX™ | 200mM | 1:100 | 2mM |
| Penicillin/streptomycin | 10,000 U/ml penicillin 10,000µg/ml streptomycin | 1:100 | 100 U/ml penicillin 100 µg/ml streptomycin |
| Amphotericin B | 250µg/ml | 1:100 | 2.5µg/ml |

**Preparation of CM2**

**[001585]** Removed prepared CM1 from refrigerator or prepare fresh CM1 as per Section 7.3 above. Removed AIM-V® from refrigerator and prepared the amount of CM2 needed by mixing prepared CM1 with an equal volume of AIM-V® in a sterile media bottle. Added 3000 IU/ml IL-2 to CM2 medium on the day of usage. Made sufficient amount of CM2 with 3000 IU/ml IL-2 on the day of usage. Labeled the CM2 media bottle with its name, the initials of the preparer, the date it was filtered/prepared, the two-week expiration date and store at 4°C until needed for tissue culture.

**Preparation of CM3**

**[001586]** Prepared CM3 on the day it was required for use. CM3 was the same as AIM-V® medium, supplemented with 3000 IU/ml IL-2 on the day of use. Prepared an amount of CM3 sufficient to experimental needs by adding IL-2 stock solution directly to the bottle or bag of AIM-V. Mixed well by gentle shaking. Label bottle with "3000 IU/ml IL-2" immediately after adding to the AIM-V. If there was excess CM3, stored it in bottles at 4°C labeled with the media name, the initials of the preparer, the date the media was prepared, and its expiration date (7 days after preparation). Discarded media supplemented with IL-2 after 7 days storage at 4°C.

**Preparation of CM4**

**[001587]** CM4 was the same as CM3, with the additional supplement of 2mM GlutaMAX™ (final concentration). For every 1L of CM3, added 10ml of 200mM GlutaMAX™. Prepared an amount of CM4 sufficient to experimental needs by adding IL-2 stock solution and GlutaMAX™ stock solution directly to the bottle or bag of AIM-V. Mixed well by gentle shaking. Labeled bottle with "3000 IU/ml IL-2 and GlutaMAX" immediately after adding to the AIM-V. If there was excess CM4, stored it in bottles at 4°C labeled with the media name, "GlutaMAX", and its expiration date (7 days after preparation). Discarded media supplemented with IL-2 after 7-days storage at 4°C.

## EXAMPLE 2: USE OF IL-2, IL-15, AND IL-21 CYTOKINE COCKTAIL

**[001588]** This example describes the use of IL-2, IL-15, and IL-21 cytokines, which serve as additional T cell growth factors, in combination with the TIL process of Examples A to G.

**[001589]** Using the processes described herein, TILs can be grown from non-small cell lung carcinoma (NSCLC) tumors in presence of IL-2 in one arm of the experiment and, in place of IL-2, a combination of IL-2, IL-15, and IL-21 in another arm at the initiation of culture. At the completion of the pre-REP, cultures were assessed for expansion, phenotype, function (CD107a+ and IFN-γ) and TCR Vβ repertoire. IL-15 and IL-21 are described elsewhere herein and in Gruijl, et al., IL-21 promotes the expansion of CD27+CD28+ tumor infiltrating lymphocytes with high cytotoxic potential and low collateral expansion of regulatory T cells, *Santegoets, S. J.,* J Transl Med., **2013,** *11:*37 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3626797/).

**[001590]** The results can show that enhanced TIL expansion (>20%), in both $CD4^+$ and $CD8^+$ cells in the IL-2, IL-15, and IL-21 treated conditions can observed relative to the IL-2 only conditions. There was a skewing towards a predominantly $CD8^+$ population with a skewed TCR Vβ repertoire in the TILs obtained from the IL-2, IL-15, and IL-21 treated cultures relative to the IL-2 only cultures. IFN-γ and CD107a were elevated in the IL-2, IL-15, and IL-21 treated TILs, in comparison to TILs treated only IL-2.

## EXAMPLE 3: QUALIFYING INDIVIDUAL LOTS OF GAMMA-IRRADIATED PERIPHERAL MONONUCLEAR CELLS

**[001591]** This Example describes a novel abbreviated procedure for qualifying individual lots of gamma-irradiated peripheral mononuclear cells (PBMCs, also known as MNC) for use as allogeneic feeder cells in the exemplary methods described herein.

**[001592]** Each irradiated MNC feeder lot was prepared from an individual donor. Each lot or donor was screened individually for its ability to expand TIL in the REP in the presence of purified anti-CD3 (clone OKT3) antibody and interleukin-2 (IL-2). In addition, each lot of feeder cells was tested without the addition of TIL.to verify that the received dose of gamma radiation was sufficient to render them replication incompetent.

### Background

**[001593]** Gamma-irradiated, growth-arrested MNC feeder cells were required for REP of TIL. Membrane receptors on the feeder MNCs bind to anti-CD3 (clone OKT3) antibody and crosslink to TIL in the REP flask, stimulating the TIL to expand. Feeder lots were prepared from the leukapheresis of whole blood taken from individual donors. The leukapheresis product was subjected to centrifugation over Ficoll-Hypaque, washed, irradiated, and cryopreserved under GMP conditions.

**[001594]** It is important that patients who received TIL therapy not be infused with viable feeder cells as this can result in Graft-Versus-Host Disease (GVHD). Feeder cells are therefore growth-arrested by dosing the cells with gamma-irradiation, resulting in double strand DNA breaks and the loss of cell viability of the MNC cells upon reculture.

### Evaluation Criteria and Experimental Set-Up

**[001595]** Feeder lots were evaluated on two criteria: 1) their ability to expand TIL in co-culture >100-fold and 2) their replication incompetency.

**[001596]** Feeder lots were tested in mini-REP format utilizing two primary pre-REP TIL lines grown in upright T25 tissue culture flasks. Feeder lots were tested against two distinct TIL lines, as each TIL line is unique in its ability to proliferate in response to activation in a REP. As a control, a lot of irradiated MNC feeder cells which has historically been shown to meet the criteria above was run alongside the test lots.

**[001597]** To ensure that all lots tested in a single experiment receive equivalent testing, sufficient stocks of the same pre-REP TIL lines were available to test all conditions and all feeder lots.

**[001598]** For each lot of feeder cells tested, there was a total of six T25 flasks: Pre-REP TIL line #1 (2 flasks); Pre-REP TIL line #2 (2 flasks); and Feeder control (2 flasks). Flasks containing TIL lines #1 and #2 evaluated the ability of the feeder lot to expand TIL. The feeder control flasks evaluated the replication incompetence of the feeder lot.

**EXPERIMENTAL PROTOCOL**

**Day -2/3, Thaw of TIL lines**

Prepared CM2 medium. Warmed CM2 in 37ºC water bath. Prepared 40 ml of CM2 supplemented with 3000IU/ml IL-2. Keep warm until use. Placed 20 ml of pre-warmed CM2 without IL-2 into each of two 50ml conical tubes labeled with names of the TIL lines used. Removed the two designated pre-REP TIL lines from LN2 storage and transferred the vials to the tissue culture room. Thawed vials by placing them inside a sealed zipper storage bag in a 37ºC water bath until a small amount of ice remains.

**[001599]** Using a sterile transfer pipet, immediately transferred the contents of vial into the 20ml of CM2 in the prepared, labeled 50ml conical tube. QS to 40ml using CM2 without IL-2 to wash cells. Centrifuged at 400 x CF for 5 minutes. Aspirated the supernatant and resuspend in 5ml warm CM2 supplemented with 3000 IU/ml IL-2.

**[001600]** Removed small aliquot (20µl) in duplicate for cell counting using an automated cell counter. Record the counts. While counting, placed the 50ml conical tube with TIL cells into a humidified 37ºC, 5% $CO_2$ incubator, with the cap loosened to allow for gas exchange. Determined cell concentration and diluted TIL to 1 x $10^6$ cells/ml in CM2 supplemented with IL-2 at 3000 IU/ml.

**[001601]** Cultured in 2ml/well of a 24-well tissue culture plate in as many wells as needed in a humidified 37ºC incubator until Day 0 of the mini-REP. Cultured the different TIL lines in separate 24-well tissue culture plates to avoid confusion and potential cross-contamination.

**Day 0, initiate Mini-REP**

**[001602]** Prepared enough CM2 medium for the number of feeder lots to be tested. (e.g., for testing 4 feeder lots at one time, prepared 800ml of CM2 medium). Aliquoted a portion of the CM2 prepared above and supplemented it with 3000 IU/ml IL-2 for the culturing of the cells.

(e.g., for testing 4 feeder lots at one time, prepare 500ml of CM2 medium with 3000 IU/ml IL-2).

**[001603]** Working with each TIL line separately to prevent cross-contamination, removed the 24-well plate with TIL culture from the incubator and transferred to the BSC.

**[001604]** Using a sterile transfer pipet or 100-1000µl Pipettor and tip, removed about 1ml of medium from each well of TIL to be used and place in an unused well of the 24-well tissue culture plate.

**[001605]** Using a fresh sterile transfer pipet or 100-1000µl Pipettor and tip, mixed remaining medium with TIL in wells to resuspend the cells and then transferred the cell suspension to a 50ml conical tube labeled with the TIL name and recorded the volume.

**[001606]** Washed the wells with the reserved media and transferred that volume to the same 50ml conical tube. Spun the cells at 400 x CF to collect the cell pellet. Aspirated off the media supernatant and resuspend the cell pellet in 2-5ml of CM2 medium containing 3000 IU/ml IL-2, volume to be used based on the number of wells harvested and the size of the pellet – volume should be sufficient to ensure a concentration of $>1.3 \times 10^6$ cells/ml.

**[001607]** Using a serological pipet, mixed the cell suspension thoroughly and recorded the volume. Removed 200µl for a cell count using an automated cell counter. While counting, placed the 50ml conical tube with TIL cells into a humidified, 5% $CO_2$, 37ºC incubator, with the cap loosened to allow gas exchange. Recorded the counts.

**[001608]** Removed the 50ml conical tube containing the TIL cells from the incubator and resuspend them cells at a concentration of $1.3 \times 10^6$ cells/ml in warm CM2 supplemented with 3000IU/ml IL-2. Returned the 50ml conical tube to the incubator with a loosened cap.

**[001609]** Repeated steps above for the second TIL line.

**[001610]** Just prior to plating the TIL into the T25 flasks for the experiment, TIL were diluted 1:10 for a final concentration of $1.3 \times 10^5$ cells/ml as per below.

**Prepare MACS GMP CD3 pure (OKT3) working solution**

**[001611]** Took out stock solution of OKT3 (1mg/ml) from 4°C refrigerator and placed in BSC. A final concentration of 30ng/ml OKT3 was used in the media of the mini-REP.

**[001612]** 600ng of OKT3 were needed for 20ml in each T25 flask of the experiment; this was the equivalent of 60µl of a 10µg/ml solution for each 20ml, or 360µl for all 6 flasks tested for each feeder lot.

**[001613]** For each feeder lot tested, made 400µl of a 1:100 dilution of 1mg/ml OKT3 for a working concentration of 10µg/ml (e.g., for testing 4 feeder lots at one time, make 1600µl of a 1:100 dilution of 1mg/ml OKT3: 16µl of 1mg/ml OKT3 + 1.584ml of CM2 medium with 3000IU/ml IL-2.)

**Prepare T25 flasks**

**[001614]** Labeled each flask and filled flask with the CM2 medium prior to preparing the feeder cells. Placed flasks into 37°C humidified 5% $CO_2$ incubator to keep media warm while waiting to add the remaining components. Once feeder cells were prepared, the components will be added to the CM2 in each flask.

TABLE 42: Solutions

| Component | Volume in co-culture flasks | Volume in control (feeder only) flasks |
|---|---|---|
| CM2 + 3000 IU/ml IL-2 | 18ml | 19ml |
| MNC: 1.3 x $10^7$/ml in CM2 + 3000IU IL-2 (final concentration 1.3 x $10^7$/flask) | 1ml | 1ml |
| OKT3: 10 µl/ml in CM2 = 3000IU IL-2 | 60µl | 60µl |
| TIL: 1.3 x $10^6$/ml in CM2 with 3000IU of IL-2 (final concentration 1.3 x $10^6$/flask) | 1ml | 0 |

**Prepare Feeder Cells**

**[001615]** A minimum of 78 x $10^6$ feeder cells were needed per lot tested for this protocol. Each 1ml vial frozen by SDBB had 100 x $10^6$ viable cells upon freezing. Assuming a 50% recovery upon thaw from LN2 storage, it was recommended to thaw at least two 1ml vials of feeder cells per lot giving an estimated 100 x $10^6$ viable cells for each REP. Alternately, if supplied in 1.8ml vials, only one vial provided enough feeder cells.

**[001616]** Before thawing feeder cells, pre-warmed approximately 50ml of CM2 without IL-2 for each feeder lot to be tested. Removed the designated feeder lot vials from LN2 storage, placed in zipper storage bag, and place on ice. Thawed vials inside closed zipper storage bag by immersing in a 37°C water bath. Removed vials from zipper bag, spray or wipe with 70% EtOH and transferred vials to BSC.

**[001617]** Using a transfer pipet immediately transferred the contents of feeder vials into 30ml of warm CM2 in a 50ml conical tube. Washed vial with a small volume of CM2 to remove any residual cells in the vial. Centrifuged at 400 x CF for 5 minutes. Aspirated the supernatant and resuspended in 4ml warm CM2 plus 3000 IU/ml IL-2. Removed 200 µl for cell counting using the Automated Cell Counter. Recorded the counts.

**[001618]** Resuspended cells at 1.3 x $10^7$ cells/ml in warm CM2 plus 3000 IU/ml IL-2. Diluted TIL cells from 1.3 x $10^6$ cells/ml to 1.3 x $10^5$ cells/ml.

**Setup Co-Culture**

**[001619]** Diluted TIL cells from 1.3 x $10^6$ cells/ml to 1.3 x $10^5$ cells/ml. Added 4.5ml of CM2 medium to a 15ml conical tube. Removed TIL cells from incubator and resuspended well using a 10ml serological pipet. Removed 0.5ml of cells from the 1.3 x $10^6$ cells/ml TIL suspension and added to the 4.5ml of medium in the 15ml conical tube. Returned TIL stock vial to incubator. Mixed well. Repeated for the second TIL line.

Transferred flasks with pre-warmed media for a single feeder lot from the incubator to the BSC. Mixed feeder cells by pipetting up and down several times with a 1ml pipet tip and transferred 1 ml (1.3 x $10^7$ cells) to each flask for that feeder lot. Added 60µl of OKT3 working stock (10µg/ml) to each flask. Returned the two control flasks to the incubator.

**[001620]** Transferred 1 ml (1.3 x $10^5$) of each TIL lot to the correspondingly labeled T25 flask. Returned flasks to the incubator and incubate upright. Did not disturb until Day 5.

**[001621]** Repeated for all feeder lots tested.

**Day 5, Media change**

Prepared CM2 with 3000 IU/ml IL-2. 10ml is needed for each flask. With a 10ml pipette, transferred 10ml warm CM2 with 3000 IU/ml IL-2 to each flask. Returned flasks to the incubator and incubated upright until Day 7. Repeated for all feeder lots tested.

**Day 7, Harvest**

**[001622]** Removed flasks from the incubator and transfer to the BSC, care as taken not to disturb the cell layer on the bottom of the flask. Without disturbing the cells growing on the bottom of the flasks, removed 10ml of medium from each test flask and 15ml of medium from each of the control flasks.

**[001623]** Using a 10ml serological pipet, resuspended the cells in the remaining medium and mix well to break up any clumps of cells. After thoroughly mixing cell suspension by pipetting, removed 200µl for cell counting. Counted the TIL using the appropriate standard operating procedure in conjunction with the automatic cell counter equipment. Recorded counts in Day 7.

**[001624]** Repeated for all feeder lots tested.

**[001625]** Feeder control flasks were evaluated for replication incompetence and flasks containing TIL were evaluated for fold expansion from Day 0 according to Table TT below.

**Day 7, Continuation of Feeder Control Flasks to Day 14**

**[001626]** After completing the Day 7 counts of the feeder control flasks, added 15ml of fresh CM2 medium containing 3000 IU/ml IL-2 to each of the control flasks. Returned the control flasks to the incubator and incubated in an upright position until Day 14.

**Day 14, Extended Non-proliferation of Feeder Control Flasks**

**[001627]** Removed flasks from the incubator and transfer to the BSC, care was taken not to disturb the cell layer on the bottom of the flask. Without disturbing the cells growing on the bottom of the flasks, removed approximately 17ml of medium from each control flasks. Using a 5ml serological pipet, resuspended the cells in the remaining medium and mixed well to break up any clumps of cells. Recorded the volumes for each flask.

**[001628]** After thoroughly mixing cell suspension by pipetting, removed 200µl for cell counting. Counted the TIL using the appropriate standard operating procedure in conjunction with the automatic cell counter equipment. Recorded counts.

**[001629]** Repeated for all feeder lots tested.

**RESULTS AND ACCEPTANCE CRITERIA**

**Results**

**[001630]** The dose of gamma irradiation was sufficient to render the feeder cells replication incompetent. All lots were expected to meet the evaluation criteria and also demonstrated a reduction in the total viable number of feeder cells remaining on Day 7 of the REP culture compared to Day 0.

**[001631]** All feeder lots were expected to meet the evaluation criteria of 100-fold expansion of TIL growth by Day 7 of the REP culture.

**[001632]** Day 14 counts of Feeder Control flasks were expected to continue the non-proliferative trend seen on Day 7.

**Acceptance Criteria**

**[001633]** The following acceptance criteria were met for each replicate TIL line tested for each lot of feeder cells

**[001634]** Acceptance was two-fold, as follows (outlined in Table 27 below).

TABLE 43: Acceptance Criteria

| Test | Acceptance criteria |
|---|---|
| Irradiation of MNC / Replication Incompetence | No growth observed at 7 and 14 days |
| TIL expansion | At least a 100-fold expansion of each TIL (minimum of 1.3 x $10^7$ viable cells) |

**[001635]** Evaluated whether the dose of radiation was sufficient to render the MNC feeder cells replication incompetent when cultured in the presence of 30ng/ml OKT3 antibody and 3000 IU/ml IL-2. Replication incompetence was evaluated by total viable cell count (TVC) as determined by automated cell counting on Day 7 and Day 14 of the REP.

**[001636]** Acceptance criteria was "No Growth," meaning the total viable cell number has not increased on Day 7 and Day 14 from the initial viable cell number put into culture on Day 0 of the REP.

**[001637]** Evaluated the ability of the feeder cells to support TIL expansion. TIL growth was measured in terms of fold expansion of viable cells from the onset of culture on Day 0 of the REP to Day 7 of the REP. On Day 7, TIL cultures achieved a minimum of 100-fold expansion, (i.e., greater than 100 times the number of total viable TIL cells put into culture on REP Day 0), as evaluated by automated cell counting.

**Contingency Testing of MNC Feeder Lots that do not meet acceptance criteria**

**[001638]** In the event that an MNC feeder lot did not meet the either of the acceptance criteria outlined above, the following steps will be taken to retest the lot to rule out simple experimenter error as its cause.

**[001639]** If there are two or more remaining satellite testing vials of the lot, then the lot was retested. If there were one or no remaining satellite testing vials of the lot, then the lot was failed according to the acceptance criteria listed above.

**[001640]** In order to be qualified, the lot in question and the control lot had to achieve the acceptance criteria above. Upon meeting these criteria, the lot was then released for use.

## EXAMPLE 4: QUALIFYING INDIVIDUAL LOTS OF GAMMA-IRRADIATED PERIPHERAL BLOOD MONONUCLEAR CELLS

**[001641]** This Example describes a novel abbreviated procedure for qualifying individual lots of gamma-irradiated peripheral blood mononuclear cells (PBMC) for use as allogeneic feeder cells in the exemplary methods described herein. This example provides a protocol for the evaluation of irradiated PBMC cell lots for use in the production of clinical lots of TIL. Each irradiated PBMC lot was prepared from an individual donor. Over the course of more than 100 qualification protocols, it was been shown that, in all cases, irradiated PBMC lots from SDBB (San Diego Blood Bank) expand TIL >100-fold on Day 7 of a REP. This modified qualification protocol was intended to apply to irradiated donor PBMC lots from SDBB which were then further tested to verify that the received dose of gamma radiation was sufficient to render them replication incompetent. Once demonstrated that they maintained replication incompetence over the course of 14 days, donor PBMC lots were considered "qualified" for usage to produce clinical lots of TIL.

### Background

**[001642]** Gamma-irradiated, growth-arrested PBMC were required for current standard REP of TIL. Membrane receptors on the PBMCs bind to anti-CD3 (clone OKT3) antibody and crosslink to TIL in culture, stimulating the TIL to expand. PBMC lots were prepared from the leukapheresis of whole blood taken from individual donors. The leukapheresis product was subjected to centrifugation over Ficoll-Hypaque, washed, irradiated, and cryopreserved under GMP conditions.

**[001643]** It is important that patients who received TIL therapy not be infused with viable PBMCs as this could result in Graft-Versus-Host Disease (GVHD). Donor PBMCs are therefore growth-arrested by dosing the cells with gamma-irradiation, resulting in double strand DNA breaks and the loss of cell viability of the PBMCs upon reculture.

### Evaluation Criteria

**[001644]** 7.2.1 Evaluation criterion for irradiated PBMC lots was their replication incompetency.

**Experimental Set-up**

**[001645]** Feeder lots were tested in mini-REP format as if they were to be co-cultured with TIL, using upright T25 tissue culture flasks. Control lot: One lot of irradiated PBMCs, which had historically been shown to meet the criterion of 7.2.1, was run alongside the experimental lots as a control. For each lot of irradiated donor PBMC tested, duplicate flasks were run.

**Experimental Protocol**

**Day 0**

**[001646]** Prepared ~90ml of CM2 medium for each lot of donor PBMC to be tested. Kept CM2 warm in 37°C water bath. Thawed an aliquot of 6 x $10^6$ IU/ml IL-2. Returned the CM2 medium to the BSC, wiping with 70% EtOH prior to placing in hood. For each lot of PBMC tested, removed about 60ml of CM2 to a separate sterile bottle. Added IL-2 from the thawed 6 x $10^6$ IU/ml stock solution to this medium for a final concentration of 3000 IU/ml. Labeled this bottle as "CM2/IL2" (or similar) to distinguish it from the unsupplemented CM2.

**Prepare OKT3**

**[001647]** Took out the stock solution of anti-CD3 (OKT3) from the 4°C refrigerator and placed in the BSC. A final concentration of 30ng/ml OKT3 was used in the media of the mini-REP. Prepared a 10µg/ml working solution of anti-CD3 (OKT3) from the 1mg/ml stock solution. Placed in refrigerator until needed.

**[001648]** For each PBMC lot tested, prepare 150µl of a 1:100 dilution of the anti-CD3 (OKT3) stock. For example, for testing 4 PBMC lots at one time, prepare 600µl of 10µg/ml anti-CD3 (OKT3) by adding 6µl of the 1mg/ml stock solution to 594µl of CM2 supplemented with 3000 IU/ml IL-2.

**Prepare Flasks**

**[001649]** Added 19ml per flask of CM2/IL-2 to the labeled T25 flasks and placed flasks into 37°C, humidified, 5% $CO_2$ incubator while preparing cells.

**Prepare Irradiate PBMC**

**[001650]** Retrieved vials of PBMC lots to be tested from LN2 storage. These were placed at -80°C or kept on dry ice prior to thawing. Placed 30ml of CM2 (without IL-2 supplement) into 50ml conical tubes for each lot to be thawed. Labeled each tube with the different lot numbers of the PBMC to be thawed. Capped tubes tightly and place in 37°C water bath prior

to use. As needed, returned 50ml conical tubes to the BSC, wiping with 70% EtOH prior to placing in the hood.

**[001651]** Removed a vial PBMC from cold storage and place in a floating tube rack in a 37°C water bath to thaw. Allowed thaw to proceed until a small amount of ice remains in the vial. Using a sterile transfer pipet, immediately transferred the contents of the vial into the 30ml of CM2 in the 50ml conical tube. Removed about 1ml of medium from the tube to rinse the vial; returned rinse to the 50ml conical tube. Capped tightly and swirl gently to wash cells.

**[001652]** Centrifuged at 400 x g for 5min at room temperature. Aspirated the supernatant and resuspend the cell pellet in 1ml of warm CM2/IL-2 using a 1000µl pipet tip. Alternately, prior to adding medium, resuspended cell pellet by dragging capped tube along an empty tube rack. After resuspending the cell pellet, brought volume to 4ml using CM2/IL-2 medium. Recorded volume.

**[001653]** Removed a small aliquot (e.g., 100µl) for cell counting using an automated cell counter. Performed counts in duplicate according to the particular automated cell counter SOP. It most likely was necessary to perform a dilution of the PBMC prior to performing the cell counts. A recommended starting dilution was 1:10, but this varied depending on the type of cell counter used. Recorded the counts.

**[001654]** Adjusted concentration of PBMC to 1.3 x $10^7$ cells/ml using CM2/IL-2 medium. Mixed well by gentle swirling or by gently aspirating up-and-down using a serological pipet.

**Set Up Culture Flasks**

**[001655]** Returned two labeled T25 flasks to the BSC from the tissue culture incubator. Returned the 10µg/ml vial of anti-CD3/OKT3 to the BSC. Added 1ml of the 1.3 x $10^7$ PBMC cell suspension to each flask. Added 60µl of the 10µg/ml anti-CD3/OKT3 to each flask. Returned capped flasks to the tissue culture incubators for 14 days of growth without disturbance. Placed anti-CD3/OKT3 vial back into the refrigerator until needed for the next lot. Repeated for each lot of PBMC to be evaluated.

**Day 14, Measurement of Non-proliferation of PBMC**

**[001656]** Returned the duplicate T25 flasks to the BSC. For each flask, using a fresh 10ml serological pipet, removed ~17ml from each of the flasks, then carefully pulled up the remaining media to measure the volume remaining in the flasks. Recorded volume.

**[001657]** Mixed sample well by pipetting up and down using the same serological pipet.

**[001658]** Removed a 200µl sample from each flask for counting. Counted cells using an automated cell counter. Repeated steps 7.4.26 – 7.4.31 for each lot of PBMC being evaluated.

**RESULTS AND ACCEPTANCE CRITERION**

**Results**

**[001659]** The dose of gamma irradiation was expected to be sufficient to render the feeder cells replication incompetent. All lots were expected to meet the evaluation criterion, demonstrating a reduction in the total viable number of feeder cells remaining on Day 14 of the REP culture compared to Day 0.

**Acceptance Criterion**

**[001660]** The following acceptance criterion were met for each irradiated donor PBMC lot tested: "No growth" – meant that the total number of viable cells on Day 14 was less than the initial viable cell number put into culture on Day 0 of the REP.

**Contingency Testing of PBMC lots which do not meet acceptance criterion.**

**[001661]** In the event than an irradiated donor PBMC lot did not meet the acceptance criterion above, the following steps were taken to retest the lot to rule out simple experimenter error as the cause of its failure. If there were two or more remaining satellite vials of the lot, then the lot was retested. If there are one or no remaining satellite vials of the lot, then the lot was failed according to the acceptance criterion above.

**[001662]** To be qualified, a PBMC lot going through contingency testing had both the control lot and both replicates of the lot in question achieve the acceptance criterion. Upon meeting this criterion, the lot was then released for use.

**EXAMPLE 5: PREPARATION OF IL-2 STOCK SOLUTION (CELLGENIX)**

**[001663]** This Example describes the process of dissolving purified, lyophilized recombinant human interleukin-2 into stock samples suitable for use in further tissue culture protocols, including all of those described in the present application and Examples, including those that involve using rhIL-2.

**Procedure**

**[001664]** Prepared 0.2% Acetic Acid solution (HAc). Transferred 29mL sterile water to a 50mL conical tube. Added lmL 1N acetic acid to the 50mL conical tube. Mixed well by inverting tube 2-3 times. Sterilized the HAc solution by filtration using a Steriflip filter

**[001665]** Prepare 1% HSA in PBS. Added 4mL of 25% HSA stock solution to 96mL PBS in a 150mL sterile filter unit. Filtered solution. Stored at 4°C. For each vial of rhIL-2 prepared, fill out forms.

**[001666]** Prepared rhIL-2 stock solution (6 x $10^6$ IU/mL final concentration). Each lot of rhIL-2 was different and required information found in the manufacturer's Certificate of Analysis (COA), such as: 1) Mass of rhIL-2 per vial (mg), 2) Specific activity of rhIL-2 (IU/mg) and 3) Recommended 0.2% HAc reconstitution volume (mL).

**[001667]** Calculated the volume of 1% HSA required for rhIL-2 lot by using the equation below:

$$\left(\frac{Vial\ Mass\ (mg)\ x\ Biological\ Activity\ \left(\frac{IU}{mg}\right)}{6x10^6\ \frac{IU}{mL}}\right) - HAc\ vol\ (mL) = 1\%\ HSA\ vol\ (mL)$$

**[001668]** For example, according to CellGenix's rhIL-2 lot 10200121 COA, the specific activity for the lmg vial is 25x$10^6$ IU/mg. It recommends reconstituting the rhIL-2 in 2mL 0.2% HAc.

$$\left(\frac{1mg\ x\ 25x10^6\ \frac{IU}{mg}}{6x10^6\ \frac{IU}{mL}}\right) - 2mL = 2.167mL\ HSA$$

**[001669]** Wiped rubber stopper of IL-2 vial with alcohol wipe. Using a 16G needle attached to a 3mL syringe, injected recommended volume of 0.2% HAc into vial. Took care to not dislodge the stopper as the needle is withdrawn. Inverted vial 3 times and swirled until all powder is dissolved. Carefully removed the stopper and set aside on an alcohol wipe. Added the calculated volume of 1% HSA to the vial.

**[001670]** Storage of rhIL-2 solution. For short-term storage (<72hrs), stored vial at 4°C. For long-term storage (>72hrs), aliquoted vial into smaller volumes and stored in cryovials at -20°C until ready to use. Avoided freeze/thaw cycles. Expired 6 months after date of preparation. Rh-IL-2 labels included vendor and catalog number, lot number, expiration date, operator initials, concentration and volume of aliquot.

## EXAMPLE 6: CRYOPRESERVATION PROCESS

**[001671]** This example describes the cryopreservation process method for TILs prepared with the abbreviated, closed procedure described in Example G using the CryoMed Controlled Rate Freezer, Model 7454 (Thermo Scientific).

**[001672]** The equipment used was as follows: aluminum cassette holder rack (compatible with CS750 freezer bags), cryostorage cassettes for 750 mL bags, low pressure (22 psi) liquid nitrogen tank, refrigerator, thermocouple sensor (ribbon type for bags), and CryoStore CS750 Freezing bags (OriGen Scientific).

**[001673]** The freezing process provides for a 0.5 °C rate from nucleation to -20 °C and 1 °C per minute cooling rate to -80 °C end temperature. The program parameters are as follows: Step 1 - wait at 4 °C; Step 2: 1.0 °C/min (sample temperature) to -4 °C; Step 3: 20.0 °C/min (chamber temperature) to -45 °C; Step 4: 10.0 °C/min (chamber temperature) to -10.0 °C; Step 5: 0.5 °C/min (chamber temperature) to -20 °C; and Step 6: 1.0 °C/min (sample temperature) to -80 °C.

## EXAMPLE 7: NSCLC TREATMENT WITH ANTI-PD-1 ANTIBODIES

Patient population:

**[001674]** Treatment naïve NSCLC or post chemotherapy but anti-PD-1/PD-L1 naïve

Treatment schedules:

**[001675]** Tumor fragment, treat with up to 4 doses of anti-PD-1/PD-L1, treat the primary refractory patients with TIL product which is cryo-preserved and ready for use upon immediate progression. Primary refractory patients may have progressed after 2 doses.

**[001676]** Relapse patients can also be treated upon progression (the timing may vary from months to years later).

**[001677]** Full strength IL-2 up to 6 doses.

**[001678]** Patient populations to further consider with the same manufacturing permutations noted earlier:

- Treatment naïve NSCLC or post chemotherapy but anti-PD-1/PD-L1 naïve
- Treatment naïve NSCLC or post chemotherapy but anti-PD-1/PD-L1 naïve who have low expression of PD-L1
- Treatment naïve NSCLC or post chemotherapy but anti-PD-1/PD-L1 naïve who have low expression of PD-L1 and/or have bulky disease at baseline (for example, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater on CT were defined as bulky; see for example, Samejima, J., *Japanese Journal of Clinical Oncology*, 45(11): 1050–10541 2015, incorporated herein by referece)

## EXAMPLE 8: GEN 3 EXEMPLARY PROCESS

**[001679]** The example provides a comparison between the Gen 2 and Gen 3 processes. This example describes the development of a robust TIL expansion platform. The modifications to the Gen 2 process reduce risk and streamline the manufacturing process by reducing the number of operator interventions, reduce the overall time of manufacturing, optimize the use of reagents, and facilitate a flexible semi-closed and semi-automated cell production process amenable to high-throughput manufacturing on a commercial scale.

**[001680]** Process Gen 2 and Gen 3 TILs are composed of autologous TIL derived from an individual patient through surgical resection of a tumor and then expanded ex vivo. The Priming First Expansion step of the Gen 3 process was a cell culture in the presence of interleukin-2 (IL-2) and the monoclonal antibody OKT3, which targets the T-cell co-receptor CD3 on a scaffold of irradiated peripheral blood mononuclear cells (PBMCs).

**[001681]** The manufacture of Gen 2 TIL products consisted of two phases: 1) pre-Rapid Expansion (Pre-REP) and 2) Rapid Expansion Protocol (REP). During the Pre-REP resected tumors were cut up into ≤ 50 fragments 2-3 mm in each dimension which were cultured with

serum-containing culture medium (RPMI 1640 media containing 10% HuSAB supplemented) and 6,000 IU/mL of Interleukin-2 (IL-2) for a period of 11 days. On day 11 TIL were harvested and introduced into the large-scale secondary REP expansion. The REP consists of activation of $\leq 200 \times 10^6$ of the viable cells from pre-REP in a co-culture of $5 \times 10^9$ irradiated allogeneic PBMCs feeder cells loaded with 150ug of monoclonal anti-CD3 antibody (OKT3) in a 5L volume of CM2 supplemented with 3000 IU/mL of rhIL-2 for 5 days. On day 16 the culture is volume reduced 90% and the cell fraction is split into multiple G-REX-500 flasks at $\geq 1 \times 10^9$ viable lymphocytes/flask and QS to 5L with CM4. TIL are incubated an additional 6 days. The REP is harvested on day 22, washed, formulated, and cryo-preserved prior to shipping at -150°C to the clinical site for infusion.

**[001682]** The manufacture of Gen 3 TIL products consisted of three phases: 1) Priming First Expansion Protocol 2) Rapid Second Expansion Protocol (also referred to as rapid expansion phase or REP) and 3) Subculture Split. To effect the Priming First Expansion TIL propagation, resected tumor was cut up into $\leq$ 120 fragments 2-3 mm in each dimension. On day 0 of the Priming First Expansion, a feeder layer of approximately $2.5 \times 10^8$ allogeneic irradiated PBMCs feeder cells loaded with OKT-3 was established on a surface area of approximately $100 cm^2$ in each of 3 100 MCS vessels. The tumor fragments were distributed among and cultured in the 3 100 MCS vessels each with 500 mL serum-containing CM1 culture medium and 6,000 IU/mL of Interleukin-2 (IL-2) and 15 ug OKT-3 for a period of 7 days. On day 7, REP was initiated by incorporating an additional feeder cell layer of approximately $5 \times 10^8$ allogeneic irradiated PBMCs feeder cells loaded with OKT-3 into the tumor fragmented culture phase in each of the 3 100 MCS vessels and culturing with 500 mL CM2 culture medium and 6,000 IU/mL IL-2 and 30 ug OKT-3. The REP initiation was enhanced by activating the entire Priming First Expansion culture in the same vessel using closed system fluid transfer of OKT3 loaded feeder cells into the 100MCS vessel. For Gen 3, the TIL scale up or split involved process steps where the whole cell culture was scaled to a larger vessel through closed system fluid transfer and was transferred (from 100 M flask to a 500 M flask) and additional 4L of CM4 media was added. The REP cells were harvested on day 16, washed, formulated, and cryo-preserved prior to shipping at -150°C to the clinical site for infusion.

**[001683]** Overall, the Gen 3 process is a shorter, more scalable, and easily modifiable expansion platform that will accommodate to fit robust manufacturing and process comparability.

**Table 44:** Comparison of Exemplary Gen 2 and Exemplary Gen 3 manufacturing process.

| Step | Process (Gen 2) | Process (Gen 3) |
|---|---|---|
| Pre REP-day 0 | Up to 50 fragments/ 1-G-Rex 100MCS - 11 days<br>In 1 L of CM1 media<br>+ IL-2 (6000 IU/mL) | Whole tumor up to 120 fragments divided evenly among up to 3 flasks. 1 flask: 1-60 fragments<br>2 flasks: 61-89 fragments<br>3 flasks 90-120 fragments<br>7 days in 500 mL of CM1 media<br>+ IL-2 (6000 IU/mL)<br>$2.5x10^8$ feeder cells/flask<br>15 ug OKT-3/flask |
| REP Initiation | Direct to REP- Day 11-<br><$200e^6$ TIL<br>(1)G-Rex 500MCS in 5L CM2 media<br>IL-2 (3000 IU/mL)<br>$5x10^9$ feeder cells<br>150ug OKT-3 | Direct to REP- Day 7-all cells TIL- same G-Rex 100MCS<br>Add 500 CM2 media<br>IL-2 (6000 IU/mL)<br>$5x10^8$ feeder cells/flask<br>30ug OKT-3/flask |
| TIL propagation or Scale up | Volume reduce and split cell fraction in up to 5 G-REX 500MCS<br>4.5L CM4 media + IL-2 (3000 IU/mL)<br>$\geq 1x10^9$ TVC / flask<br>Split day 16 | Each G-REX 100MCS(1L) transfers to 1 G-REX 500MCS<br>Add 4L CM4 media +IL-2 (3000 IU/mL)<br>Scale up on day 9 to 11 |
| Harvest | Harvest day 22,<br>LOVO-automated cell washer | Harvest day 16<br>LOVO- automated cell washer |
| Final formulation | Cryopreserved Product<br>300IU/ml IL2- CS10 in $LN_2$, multiple aliquots | Cryopreserved product<br>300IU/ml IL-2-CS10 in $LN_2$,<br>multiple aliquots |
| **Process time** | **22 days** | **16 days** |

**[001684]** On day 0, for both processes, the tumor was washed 3 times and the fragments were randomized and divided into two pools; one pool per process. For the Gen 2 Process, the fragments were transferred to one GREX 100MCS flask with 1 L of CM1 media containing 6,000IU/mL rhIL-2. For the Gen 3 Process, fragments were transferred to one GREX100MCS flask with 500 mL of CM1 containing 6,000IU/mL rhIL-2, 15 ug OKT-3 and $2.5 \times 10^8$ feeder cells. Seeding of TIL for Rep initiation day occurred on different days according to each process. For the Gen 2 Process, in which the G-REX 100MCS flask was

90% volume reduced, collected cell suspension was transferred to a new G-REX 500MCS to start REP initiation on day 11 in CM2 media containing IL-2 (3000 IU/mL), plus 5e9 feeder cells and OKT-3 (30 ng/mL). Cells were expanded and split on day 16 into multiple GREX 500 MCS flasks with CM4 media with IL-2 (3000 IU/mL) per protocol. The culture was then harvested and cryopreserved on day 22 per protocol. For the Gen 3 process, the REP initiation occurred on day 7, in which the same G-REX 100MCS used for REP initiation. Briefly, 500 mL of CM2 media containing IL-2 (6000 IU/mL) and 5 x $10^8$ feeder cells with 30ug OKT-3 was added to each flask. On day 9-11 the culture was scaled up. The entire volume of the G-Rex100M (1L) was transferred to a G-REX 500MCS and 4L of CM4 containing IL-2 (3000 IU/mL) was added. Flasks were incubated 5 days. Cultures were harvested and cryopreserved on Day 16.

**[001685]** Three different tumors were included in the comparison, two lung tumors (L4054 and L4055) and one melanoma tumor (M1085T).

**[001686]** CM1 (culture media 1), CM2 (culture media 2), and CM4 (culture media 4) media were prepared in advance and held at 4°C for L4054 and L4055. CM1 and CM2 media were prepared without filtration to compare cell growth with and without filtration of media.

**[001687]** Media was warmed at 37°C up to 24 hours in advance for L4055 tumor on REP initiation and scale-up.

**Results Summary**

**[001688]** Gen 3 fell within 30% of Gen 2 for total viable cells achieved. Gen 3 final product exhibited higher production of INF-γ after restimulation. Gen 3 final product exhibited increased clonal diversity as measured by total unique CDR3 sequences present. Gen 3 final product exhibited longer mean telomere length.

**Results achieved**

**Cell count and % viability:**

**[001689]** Pre REP and REP expansion on Gen 2 and Gen 3 processes followed details described above.

**[001690]** **Table 45: Pre-REP cell counts.** For each tumor, the two pools contained equal number of fragments. Due to the small size of tumors, the maximum number of fragments per flask was not achieved. Total pre-REP cells (TVC) were harvested and counted at day 11 for the Gen 2 process and at day 7 for the Gen 3 process. To compare the two pre-

REP arms, the cell count was divided over the number of fragments provided in the culture in order to calculate an average of viable cells per fragment. As indicated in the table below, the Gen 2 process consistently grew more cells per fragment compared to the Gen 3 Process. An extrapolated calculation of the number of TVC expected for Gen 3 process at day 11, which was calculated dividing the pre-REP TVC by 7 and then multiply by 11.

**Table 45: pre-REP cell counts**

| Tumor ID | L4054 | | L4055* | | M1085T | |
|---|---|---|---|---|---|---|
| Process | Gen 2 | Gen 3 | Gen 2 | Gen 3 | Gen 2 | Gen 3 |
| pre-REP TVC | 1.42E+08 | 4.32E+07 | 2.68E+07 | 1.38E+07 | 1.23E+07 | 3.50E+06 |
| Number of fragments | 21 | 21 | 24 | 24 | 16 | 16 |
| Average TVC per fragment at pre-REP | 6.65E+06 | 2.06E+06 | 1.12E+06 | 5.75E+05 | 7.66E+05 | 2.18E+05 |
| Gen 3 extrapolated value at pre REP day 11 | N/A | 6.79E+07 | N/A | 2.17E+07 | N/A | 5.49E+06 |

*** L4055, unfiltered media.**

**[001691] Table 46: Total viable cell count and fold expansion on TIL final product:** For the Gen 2 and Gen 3 processes, TVC was counted per process condition and percent viable cells was generated for each day of the process. On harvest, day 22 (Gen 2) and day 16 (Gen 3) cells were collected and the TVC count was established. The TVC was then divided by the number of fragments provided on day 0, to calculate an average of viable cells per fragment. Fold expansion was calculated by dividing harvest TVC by over the REP initiation TVC. As exhibited in the table, comparing Gen 2 and the Gen 3, fold expansions were similar for L4054; in the case of L4055, the fold expansion was higher for the Gen 2 process. Specifically, in this case, the media was warmed up 24 in advance of REP initiation day. A higher fold expansion was also observed in Gen 3 for M1085T. An extrapolated calculation of the number of TVC expected for Gen 3 process at day 22, which was calculated dividing the REP TVC by 16 and then multiply by 22.

**Table 46: Total viable cell count and fold expansion on TIL final product**

| Tumor ID | L4054 | | L4055 | | M1085T | |
|---|---|---|---|---|---|---|
| Process | Gen 2 | Gen 3 | Gen 2 | Gen 3 | Gen 2 | Gen 3 |
| # Fragments | 21 | 21 | 24 | 24 | 16 | 16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| TVC /fragment (at Harvest) | 3.18E+09 | 8.77E+08 | 2.30E+09 | 3.65E+08 | 7.09E+08 | 4.80E+08 |
| REP initiation | 1.42E+08 | 4.32E+07 | 2.68E+07 | 1.38E+07 | 1.23E+07 | 3.50E+06 |
| Scale up | 3.36E+09 | 9.35E+08 | 3.49E+09 | 8.44E+08 | 1.99E+09 | 3.25E+08 |
| Harvest | 6.67E+10 | 1.84E+10 | 5.52E+10 | 8.76E+09 | 1.13E+10 | 7.68E+09 |
| Fold Expansion Harvest/ REP initiation | **468.4** | **425.9** | **2056.8** | **634.6** | **925.0** | **2197.2** |
| Gen 3 extrapolated value at REP harvest day 22 | **N/A** | **2.53E+10** | **N/A** | **1.20E+10** | **N/A** | **1.06E+10** |

*** L4055, unfiltered media.**

**[001692]** **Table 47: %Viability of TIL final product:** Upon harvest, the final TIL REP products were compared against release criteria for % viability. All of the conditions for the Gen 2 and Gen 3 processes surpassed the 70% viability criterion and were comparable across processes and tumors.

**Table 47: % Viability of REP**

| Tumor ID | L4054 | | L4055 | | M1085T | |
|---|---|---|---|---|---|---|
| Process | Gen 2 | Gen 3 | Gen 2 | Gen 3 | Gen 2 | Gen 3 |
| REP initiation | 98.23% | 97.97% | 97.43% | 92.03% | 81.85% | 68.27% |
| Scale up | 94.00% | 93.57% | 90.50% | 95.93% | 78.55% | 71.15% |
| Harvest | 87.95% | 89.85% | 87.50% | 86.70% | 86.10% | 87.45% |

**[001693]** **Table 48: Estimate cell count per additional flask for Gen 3 process**. Due to the number of fragments per flask below the maximum required number, an estimated cell count at harvest day was calculated for each tumor. The estimation was based on the expectation that clinical tumors were large enough to seed 2 or 3 flasks on day 0.

**Table 49: Extrapolated estimate cell count calculation to full scale 2 and 3 flask on Gen 3 Process**

| Tumor ID | L4054 | | L4055 | | M1085T | |
|---|---|---|---|---|---|---|
| Gen 3 Process | 2 flasks | 3 Flasks | 2 flasks | 3 Flasks | 2 flasks | 3 Flasks |

| | | | | | | |
|---|---|---|---|---|---|---|
| Estimate Harvest | 3.68E+10 | 5.52E+10 | 1.75E+10 | 2.63E+10 | 1.54E+10 | 2.30E+10 |

**IMMUNOPHENOTYPING:**

**Phenotypic markers comparison on TIL final product:**

**[001694]** Three tumors L4054, L4055, and M1085T underwent TIL expansion in both the Gen 2 and Gen 3 processes. Upon harvest, the REP TIL final products were subjected to flow cytometry analysis to test purity, differentiation, and memory markers. For all the conditions the percentage of TCR a/b+ cells was over 90%.

**[001695]** TIL harvested from the Gen 3 process showed a higher expression of CD8 and CD28 compared to TIL harvested from the Gen 2 process. The Gen 2 process showed a higher percentage of CD4+. **See, Figure 3 (A, B, C)**.

**Memory markers comparison on TIL final product:**

**[001696]** TIL harvested from the Gen 3 process showed a higher expression on central memory compartments compared to TIL from the Gen 2 process. **See, Figure 4 (A, B, C)**.

**Activation and exhaustion markers comparison on TIL final product:**

**[001697]** Activation and exhaustion marker were analyzed in TIL from two, tumors L4054 and L4055 to compare the final TIL product by from the Gen 2 and Gen 3 TIL expansion processes. Activation and exhaustion markers were comparable between the Gen 2 and Gen 3 processes**. See, Figure 5 (A, B); Figure 6 (A, B).**

**INTERFERON GAMMA SECRETION UPON RESTIMULATION:**

**[001698]** On harvest day, day 22 for Gen 2 and day 16 for Gen 3, TIL underwent an overnight restimulation with coated anti -CD3 plates for L4054 and L4055. The restimulation on M1085T was performed using anti-CD3, CD28, and CD137 beads. Supernatant was collected after 24 hours of the restimulation in all conditions and the supernatant was frozen. IFNγ analysis by ELISA was assessed on the supernatant from both processes at the same time using the same ELISA plate. Higher production of IFNγ from the Gen 3 process was observed in the three tumors analyzed. **See, Figure 7 (A, B, C).**

**MEASUREMENT OF IL-2 LEVELS IN CULTURE MEDIA:**

**[001699]** To compare the IL-2 consumption between Gen 2 and Gen 3 process, cell supernatant was collected on REP initiation, scale up, and harvest day, on tumor L4054 and L4055. The quantity of IL-2 in cell culture supernatant was measured by Quantitate ELISA Kit from R&D. The general trend indicates that the IL-2 concentration remains higher in the Gen 3 process when compared to the Gen 2 process. This is likely due to the higher concentration of IL-2 on REP initiation (6000 IU/mL) for Gen 3 coupled with the carryover of the media throughout the process. **See, Figure 8 (A, B).**

## METABOLIC SUBSTRATE AND METABOLITE ANALYSIS

**[001700]** The levels of metabolic substrates such as D-glucose and L-glutamine were measured as surrogates of overall media consumption. Their reciprocal metabolites, such lactic acid and ammonia, were measured. Glucose is a simple sugar in media that is utilized by mitochondria to produce energy in the form of ATP. When glucose is oxidized, lactic acid is produced (lactate is an ester of lactic acid). Lactate is strongly produced during the cells exponential growth phase. High levels of lactate have a negative impact on cell culture processes. **See, Figure 9 (A, B).**

**[001701]** Spent media for L4054 and L4055 was collected at REP initiation, scale up, and harvest days for both process Gen 2 and Gen 3. The spent media collection was for Gen 2 on Day 11, day 16 and day 22; for Gen 3 was on day 7, day 11 and day 16. Supernatant was analyzed on a CEDEX Bio-analyzer for concentrations of glucose, lactic acid, glutamine, glutamax, and ammonia.

**[001702]** L- glutamine is an unstable essential amino acid required in cell culture media formulations. Glutamine contains an amine, and this amide structural group can transport and deliver nitrogen to cells. When L-glutamine oxidizes, a toxic ammonia by-product is produced by the cell. To counteract the degradation of L-glutamine the media for the Gen 2 and Gen 3 processes was supplemented with Glutamax, which is more stable in aqueous solutions and does not spontaneously degrade. In the two tumor lines, the Gen 3 arm showed a decrease in L-glutamine and Glutamax during the process and an increase in ammonia throughout the REP. In the Gen 2 arm a constant concentration of L-glutamine and Glutamax, and a slight increase in the ammonia production was observed. The Gen 2 and Gen 3 processes were comparable at harvest day for ammonia and showed a slight difference in L-glutamine degradation. **See, Figure 10 (A, B, C).**

**TELOMERE REPEATS BY FLOW – FISH:**

**[001703]** Flow-FISH technology was used to measure the average length of the telomere repeat on L4054 and L4055 under Gen 2 and Gen 3 process. The determination of a relative telomere length (RTL) was calculated using Telomere PNA kit/FITC for flow cytometry analysis from DAKO. Gen 3 showed comparable telomere length to Gen 2.

**CD3 Analysis**

**[001704]** To determine the clonal diversity of the cell products generated in each process, TIL final product harvested for L4054 and L4055, were sampled and assayed for clonal diversity analysis through sequencing of the CDR3 portion of the T-cell receptors.

**[001705]** Table 50: Comparison of Gen 2 and Gen3 of percentage shared unique CDR3 sequences on L4054 on TIL harvested cell product. 199 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 97.07% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

**Table 50: Comparison of shared uCDR3 sequences between Gen 2 and Gen 3 processes on L4054.**

| # uCDR3 (% Overlap) | All uCDR3's | | Top 80% uCDR3's | |
|---|---|---|---|---|
| | Gen 2 | Gen 3 | Gen 2 | Gen 3 |
| Gen 2-L4054 | 8915 | 4355 **(48.85%)** | 205 | 199 **(97.07%)** |
| Gen 3-L4054 | - | 18130 | - | 223 |

**[001706]** **Table 51: Comparison of Gen 2 and Gen3 of percentage shared unique CDR3 sequences on L4055 on TIL harvested cell product.** 1833 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 99.45% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

**Table 51: Comparison of shared uCDR3 sequences between Gen 2 and Gen 3 processes on L4055.**

| # uCDR3 (% Overlap) | All uCDR3's | | Top 80% uCDR3's | |
|---|---|---|---|---|
| | Gen 2 | Gen 3 | Gen 2 | Gen 3 |
| Gen 2-L4055 | 12996 | 6599 **(50.77%)** | 1843 | 1833 **(99.45%)** |
| Gen 3-L4055 | - | 27246 | - | 2616 |

**[001707]** CM1 and CM2 media was prepared in advanced without filtration and held at 4 degree C until use for tumor L4055 to use on Gen 2 and Gen 3 process.

**[001708]** Media was warmed up at 37 degree C for 24 hours in advance for tumor L4055 on REP initiation day for Gen 2 and Gen 3 process.

**[001709]** LDH was not measured in the supernatants collected on the processes.

**[001710]** M1085T TIL cell count was executed with K2 cellometer cell counter.

**[001711]** On tumor M1085T, samples were not available such as supernatant for metabolic analysis, TIL product for activation and exhaustion markers analysis, telomere length and CD3 - TCR vb Analysis.

## CONCLUSIONS

**[001712]** This example compares 3 independent donor tumors tissue in terms of functional quality attributes, plus extended phenotypic characterization and media consumption among Gen 2 and Gen 3 processes.

**[001713]** Gen 2 and Gen 3 pre-REP and REP expansion comparison were evaluated in terms of total viable cells generated and viability of the total nucleated cell population. TVC cell doses at harvest day was not comparable between Gen 2 (22 days) and Gen 3 (16 days). Gen 3 cell doses were lower than Gen 2 at around 40% of total viable cells collected at harvest.

**[001714]** An extrapolated cell number was calculated for Gen 3 process assuming the pre-REP harvest occurred at day 11 instead day 7 and REP Harvest at Day 22 instead day 16. In both cases shows closer number on TVC compared to Gen 2 process, indicating that the early activation could allow an overall better performance on TIL growth. Table 4 and 5 bottom row.

**[001715]** In the case of extrapolated value for extra flasks (2 or 3) on Gen 3 process assuming a bigger size of tumor processed, and reaching the maximum number of fragments required per process as described. It was observed that a similar dose can be reachable on TVC at Day 16 Harvest for Gen 3 process compared to Gen 2 process at Day 22. This observation is important and indicates an early activation of the culture can allow better performance of TIL in less processing time.

**[001716]** Gen 2 and Gen 3 pre-REP and REP expansion comparison were evaluated in terms of total viable cells generated and viability of the total nucleated cell population. TVC cell doses at harvest day was not comparable between Gen 2 (22 days) and Gen 3 (16 days). Gen 3 cell doses were lower than Gen 2 at around 40% of total viable cells collected at harvest.

**[001717]** In terms of phenotypic characterization, a higher CD8+ and CD28+ expression was observed on three tumors on Gen 3 process compared to Gen 2 process. This data indicates the Gen 3 process has improved attributes of final TIL product compared to Gen 2.

**[001718]** Gen 3 process showed slightly higher central memory compartments compared to Gen 2 process.

**[001719]** Gen 2 and Gen 3 process showed comparable activation and exhaustion markers, despite the shorter duration of the Gen 3 process.

**[001720]** IFN gamma (IFNγ) production was 3 times higher on Gen 3 final product compared to Gen 2 in the three tumors analyzed. This data indicates the Gen 3 process generated a highly functional and more potent TIL product as compared to the Gen 2 process, possibly due to the higher expression of CD8 and CD28 expression on Gen 3. Phenotypic characterization suggested positive trends in Gen 3 toward CD8+, CD28+ expression on three tumors compared to Gen 2 process.

**[001721]** Telomere length on TIL final product between Gen 2 and Gen 3 were comparable.

**[001722]** Glucose and Lactate levels were comparable between Gen 2 and Gen 3 final product, suggesting the levels of nutrients on the media of Gen 3 process were not affected due to the non-execution of volume reduction removal in each day of the process and less volume media overall in the process, compared to Gen 2.

**[001723]** Overall Gen 3 process showed a reduction almost two times of the processing time compared to Gen 2 process, which would yield a substantial reduction on the cost of goods (COGs) for TIL product expanded by the Gen 3 process.

**[001724]** IL-2 consumption indicates a general trend of IL-2 consumption on Gen 2 process, and in Gen 3 process IL-2 was higher due to the non-removal of the old media.

**[001725]** The Gen 3 process showed a higher clonal diversity measured by CDR3 TCRab sequence analysis.

**[001726]** The addition of feeders and OKT-3 on day 0 of the pre-REP allowed an early activation of TIL and overall a better growth TIL performance using the Gen 3 process.

**[001727]** Table 52 describes various embodiments and outcomes for the Gen 3 process as compared to the current Gen 2 process:

**Table 52: Exemplary Gen 3 process.**

| Step | Process Gen 2 | Process Gen 3-Optimized |
|---|---|---|
| **Pre REP-day 0** | ≤50 fragments<br>1X G-Rex 100MCS<br>1L media<br>IL-2 (6000IU/mL)<br>11 days | ≤240 fragments<br>≤60 fragments/flask<br>≤4 flasks<br>≤2L media (500mL/flask)<br>IL-2 (6000IU/mL)<br>$2.5x10^8$ feeder cells/flask<br>15ug OKT3/flask |
| **REP Initiation** | Fresh TIL direct to REP<br>Day 11<br>$\leq 200e^6$ viable cells<br>$5x10^9$ feeder cells<br>G-Rex 500MCS<br>5L CM2 media + IL-2 (3000 IU/mL)<br>150ug OKT3 | Fresh TIL direct to REP<br>Day 7<br>Activate entire culture<br>$5x10^8$ feeder cells<br>30 ug OKT3/flask<br>G-Rex 100MCS<br>500mL media+ IL-2(6000IU/mL) |
| **TIL Sub-culture or Scale up** | ≤5 G-REX 500MCS<br>≤1x10 viable cells/ flask<br>5L/flask<br>Day 16 | ≤4 G-REX 500MCS<br>Scale up entire culture<br>4L/flask<br>Day 10-11 |
| **Harvest** | Harvest Day 22,<br>LOVO-automated cell washer<br>2 wash cycles | Harvest Day 16<br>LOVO- automated cell washer<br>5 wash cycles |
| **Final formulation** | Cryopreserved Product<br>300IU/ml IL2- CS10 in $LN_2$, multiple aliquots | Cryopreserved product<br>300IU/ml IL-2-CS10 in $LN_2$,<br>multiple aliquots |
| **Process time** | **22 days** | **16 days** |

## EXAMPLE 9: AN EXEMPLARY EMBODIMENT OF SELECTING AND EXPANDING PBLS FROM PBMCS IN CLL PATIENTS.

**[001728]** PBMCs are collected from patients and either frozen for later use, or used fresh. Enough volume of peripheral blood is collected to yield at least about 400,000,000 ($400 \times 10^6$) PBMCs for starting material in the method of the present invention. On Day 0 of the method, IL-2 at $6x10^6$ IU/mL is either prepared fresh or thawed, and stored at 4°C or on ice until ready to use. 200 mL of CM2 medium is prepared by combining 100 mL of CM1 medium (containing GlutaMAX®), then diluting it with 100 mL (1:1) with AIM-V to make CM2. The CM2 is protected from light, and sealed tightly when not in use.

**[001729]** All of the following steps are performed under sterile cell culture conditions. An aliquot of CM2 is warmed in a 50mL conical tube in a 37°C water bath for use in thawing and/or washing a frozen PBMC sample. If a frozen PBMC sample is used, the sample is removed from freezer storage and kept on dry ice until ready to thaw. When ready to thaw the PBMC cryovial, 5 mL of CM2 medium is placed in a sterile 50 mL conical tube. The PBMC sample cryovial is placed in a 37°C water bath until only a few ice crystals remain. Warmed CM2 medium is added, dropwise, to the sample vial in a 1:1 volume ratio of sample:medium (about 1 mL). The entire contents is removed from the cryovial and transferred to the remaining CM2 medium in the 50 mL conical tube. An additional 1-2 mL of CM2 medium is used to rinse the cryovial and the entire contents of the cryovial is removed and transferred to the 50 mL conical tube. The volume in the conical tube is then adjusted with additional CM2 medium to 15 mL, and swirled gently to rinse the cells. The conical tube is then centrifuged at 400g for 5 minutes at room temperature in order to collect the cell pellet.

**[001730]** The supernatant is removed from the pellet, the conical tube is capped, and then the cell pellet is disrupted by, for example, scraping the tube along a rough surface. About 1mL of CM2 medium is added to the cell pellet, and the pellet and medium are aspirated up and down 5-10 times with a pipette to break up the cell pellet. An additional 3-5 mL of CM2 medium is added to the tube and mixed via pipette to suspend the cells. At this point, the volume of the cell suspension is recorded. Remove 100 uL of the cell suspension from the tube for cell counting with an automatic cell counter, for example, a Nexcelom Cellometer K2. Determine the number of live cells in the sample and record.

**[001731]** Reserve a minimum of 5 x $10^6$ cells for phenotyping and other characterization experiments. Spin the reserved cells at 400g for 5 minutes at room temperature to collect the cell pellet. Resuspend the cell pellet in freezing medium (sterile, heat-inactivated FBS containing 20% DMSO). Freeze one or two aliquots of the reserved cells in freezing medium, and slow-freeze the aliquots in a cell freezer (Mr. Frosty) in a -80°C freezer. Transfer to liquid nitrogen storage after a minimum of 24 hours at -80°C.

**[001732]** For the following steps, use pre-cooled solutions, work quickly, and keep the cells cold. The next step is to purify the T-cell fraction of the PBMC sample. This is completed using a Pan T-cell Isolation Kit (Miltenyi, catalog # 130-096-535). Prepare the cells for purification by washing the cells with a sterile-filtered wash buffer containing PBS, 0.5% BSA, and 2mM EDTA at pH 7.2. The PBMC sample is centrifuged at 400g for 5 minutes to collect the cell pellet. The supernatant is aspirated off and the cell pellet is resuspended in 40 uL of wash buffer for every $10^7$ cells. Add 10 uL of Pan T Cell Biotin-Antibody Cocktail for every $10^7$ cells. Mix well and incubate for 5 minutes in refrigerator or on ice. Add 30 uL of wash buffer for every $10^7$ cells. Add 20 uL of Pan T-cell MicroBead Cocktail for every $10^7$ cells. Mix well and incubate for 10 minutes in refrigerator or on ice. Prepare an LS column and magnetically separate cells from the microbeads. The LS column is placed in the QuadroMACS magnetic field. The LS column is washed with 3 mL of cold wash buffer, and the wash is collected and discarded. The cell suspension is applied to the column and the flow-through (unlabeled cells) is collected. This flow-through is the enriched T-cell fraction (PBLs). Wash the column with 3 mL of wash buffer and collect the flow-through in the same tube as the initial flow-through. Cap the tube and place on ice. This is the T-cell fraction, or PBLs. Remove the LS column from the magnetic field, wash the column with 5 mL of wash buffer, and collect the non-T-cell fraction (magnetically labeled cells) into another tube. Centrifuge both fractions at 400g for 5 minutes to collect the cell pellets. Supernatants are aspirated from both samples, disrupt the pellet, and resuspend the cells in 1 mL of CM2 medium supplemented with 3000 IU/mL IL-2 to each pellet, and pipette up and down 5-10 times to break up the pellets. Add 1-2 mL of CM2 to each sample, and mix each sample well, and store in tissue culture incubator for next steps. Remove about a 50 uL aliquot from each sample, count cells, and record count and viability.

**[001733]** The T-cells (PBLs) are then cultured with Dunabeads™ Human T-Expander CD3/CD28. A stock vial of Dynabeads is vortexed for 30 seconds at medium spead. A required aliquot of beads is removed from the stock vial into a sterile 1.5 mL microtube. The

beads are washed with bead wash solution by adding 1 mL of bead wash to the 1.5 mL microtube containing the beads. Mix gently. Place the tube onto the DynaMag™-2 magnet and let sit for 30 minutes while beads draw toward the magnet. Aspirate the wash solution off the beads and remove tube from the magnet. 1mL of CM2 medium supplemented with 3000 IU/mL IL-2 is added to the beads. The entire contents of the microtube is transferred to a 15 or 50 mL conical tube. Bring the beads to a final concentration of about 500,000/mL using CM2 medium with IL-2.

**[001734]** The T-cells (PBLs) and beads are cultured together as follows. On day 0: In a G-Rex 24 well plate, in a total of 7mL per well, add 500,000 T-cells, 500,000 CD3/CD28 Dynabeads, and CM2 supplemented with IL-2. The G-Rex plate is placed into a humidified 37°C, 5% $CO_2$ incubator until the next step in the process (on Day 4). Remaining cells are frozen in CS10 cryopreservation medium using a Mr. Frosty cell freezer. The non-T-cell fraction of cells are frozen in CS10 cryopreservation medium using a Mr. Frosty cell freezer. On day 4, medium is exchanged. Half of the medium (about 3.5mL) is removed from each well of the G-rex plate. A sufficient volume (about 3.5mL) of CM4 medium supplemented with 3000 IU/mL IL-2 warmed to 37°C is added to replace the medium removed from each sample well. The G-rex plate is returned to the incubator.

**[001735]** On day 7, cells are prepared for expansion by REP. The G-rex plate is removed from the incubator and half of medium is removed from each well and discarded. The cells are resuspended in the remaining medium and transferred to a 15 mL conical tube. The wells are washed with 1 mL each of CM4 supplemented with 3000 IU/mL IL-2 warmed to 37°C and the wash medium is transferred to the same 15 mL tube with the cells. A representative sample of cells is removed and counted using an automated cell counter. If there are less than $1x10^6$ live cells, the Dynabead expansion process at Day 0 is repeated. The remainder of the cells are frozen for back-up expansion or for phenotyping and other characterization studies. If there are $1x10^6$ live cells or more, the REP expansion is set up in replicate according to the protocol from Day 0. Alternatively, with enough cells, the expansion may be set up in a G-rex 10M culture flask using $10\text{-}15x10^6$ PBLs per flask and a 1:1 ratio of Dynabeads:PBLs in a final volume of 100mL/well of CM4 medium supplemented with 3000 IU/mL IL-2. The plate and/or flask is returned to the incubator. Excess PBLs may be aliquotted and slow-frozen in a Mr. Frosty cell freezer in a -80°C freezer, and the transferred to liquid nitrogen storage after a minimum of 24 hours at -

80°C. These PBLs may be used as back-up samples for expansion or for phenotyping or other characterization studies.

**[001736]** On Day 11, the medium is exchanged. Half of the medium is removed from either each well of the G-rex plate or the flask and replaced with the same amount of fresh CM4 medium supplemented with 3000 IU/mL IL-2 at 37°C.

**[001737]** On Day 14, the PBLs are harvested. If the G-rex plate is used, about half of the medium is removed from each well of the plate and discarded. The PBLs and beads are suspended in the remaining medium and transferred to a sterile 15 mL conical tube (Tube 1). The wells are washed with 1-2 mL of fresh AIM-V medium warmed to 37°C, and the wash is transferred to Tube 1. Tube 1 is capped and placed in the DynaMag™-15 Magnet for 1 minute to allow the beads to be drawn to the magnet. The cell suspension is transferred into a new 15 mL tube (Tube 2), and the beads are washed with 2mL of fresh AIM-V at 37°C. Tube 1 is placed back in the magnet for an additional 1 minute, and the wash medium is then transferred to Tube 2. The wells may be combined if desired, after the final washing step. Remove a representative sample of cells and count, record count and viability. Tubes may be placed in the incubator while counting. Additional AIM-V medium may be added to the Tube 2 if cells appear very dense. If a flask is used, the volume in the flask should be reduced to about 10 mL. The contents of the flask is mixed and transferred to a 15 mL conical tube (Tube A). The flask is washed with 2mL of the AIM-V medium as described above and the wash medium is also transferred to Tube A. Tube A is capped and placed in the DynaMag™-15 Magnet for 1 minute to allow the beads to be drawn to the magnet. The cell suspension is transferred into a new 15 mL tube (Tube B), and the beads are washed with 2mL of fresh AIM-V at 37°C. Tube A is placed back in the magnet for an additional 1 minute, and the wash medium is then transferred to Tube B. The wells may be combined if desired, after the final washing step. Remove a representative sample of cells and count, record count and viability. Tubes may be placed in the incubator while counting. Additional AIM-V medium may be added to the Tube B if cells appear very dense. Cells may be used fresh or frozen in CS10 preservation medium at desired concentrations.

## EXAMPLE 10: A PHASE 2, MULTICENTER STUDY OF AUTOLOGOUS TUMOR INFILTRATING LYMPHOCYTES IN PATIENTS WITH SOLID TUMORS

**[001738]** **STUDY DESIGN**

**[001739]** Overview

**[001740]** This example describes a prospective, open-label, multi-cohort, non-randomized, multicenter Phase 2 study evaluating ACT using TIL in combination with pembrolizumab or TIL as a single therapy, using TILs prepared as described in the present application as well as in this example.

**[001741]** Objectives:

**[001742]** *Primary:*

**[001743]** To evaluate the efficacy of autologous TIL in combination with pembrolizumab in MM, HNSCC, or NSCLC patients or TIL as a single therapy in relapsed or refractory (r/r) NSCLC patients, who had previously progressed on or after treatment with CPIs, as determined by objective response rate (ORR), using the Response Evaluation Criteria in Solid Tumors (RECIST 1.1), as assessed by Investigator.

**[001744]** To characterize the safety profile of TIL in combination with pembrolizumab in MM, HNSCC, and NSCLC patients or TIL as a single therapy in r/r NSCLC patients as measured by the incidence of Grade $\geq 3$ treatment-emergent adverse events (TEAEs).

**[001745]** *Secondary:*

**[001746]** To further evaluate the efficacy of autologous TIL in combination with pembrolizumab in MM, HNSCC, and NSCLC patients or TIL as a single therapy in r/r NSCLC patients using complete response (CR) rate, duration of response (DOR), disease control rate (DCR), progression-free survival (PFS) using RECIST 1.1, as assessed by Investigator, and overall survival (OS).

**[001747]** Cohorts:

**[001748]** Cohort 1A: TIL therapy in combination with pembrolizumab in patients with Stage IIIC or Stage IV unresectable or MM with $\leq 3$ prior lines of systemic therapy excluding immunotherapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001749]** Cohort 2A: TIL therapy in combination with pembrolizumab in patients with advanced, recurrent or metastatic HNSCC (*e.g.*, Stages T1N1-N2B, T2-4N0-N2b) with $\leq 3$ prior lines of systemic therapy, excluding immunotherapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001750]** Cohort 3A: TIL therapy in combination with pembrolizumab in patients with locally advanced or metastatic (Stage III– IV) NSCLC with ≤3 prior lines of systemic therapy, excluding immunotherapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001751]** Cohort 3B: TIL therapy as a single agent in patients Stage III or Stage IV NSCLC who have previously received systemic therapy with CPIs (*e.g.*, anti-PD-1/anti-PD-L1) as part of ≤ 3 prior lines of systemic therapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001752]** Patients in Cohorts 3A and 3B (NSCLC) with oncogene-driven tumors with available effective targeted therapy must have received at least one line of targeted therapy.

**[001753]** All patients received autologous cryopreserved TIL therapy (with or without pembrolizumab, depending on cohort assignment), preceded by a nonmyeloablative lymphodepletion (NMA-LD) preconditioning regimen consisting of cyclophosphamide and fludarabine. Following TIL infusion, up to 6 IV interleukin-2 (IL-2) doses maximum were administered.

**[001754]** The following general study periods took place in all 4 cohorts, unless specified otherwise.

**[001755]** Screening and Tumor Resection: Up to 4 weeks (28 days) from study entry; manufacturing of the TIL Product: approximately ≤22 days from tumor resection; and treatment period, as discussed below.

**[001756]** Treatment Period (Cohorts 1A, 2A, and 3A): up to 2 years, including NMA-LD (7 days), TIL infusion (1 day) followed by IL-2 administrations (1 to 4 days).Patients receive a single infusion of pembrolizumab after the completion of their tumor resection for TIL production and baseline scans but before the initiation of the NMA-LD regimen. The next dose of pembrolizumab will be no earlier than following the completion of IL-2 and continue Q3W ± 3 days thereafter for ≤ 2 years (24 months) or until disease progression or unacceptable toxicity, whichever occurs first. The end-of-treatment (EOT) visit occurred within 30 days after the last dose of pembrolizumab. The visit could be combined with end-of-assessment (EOA) visit if applicable (*e.g.*, pembrolizumab discontinuation occurred at disease progression or at the start of new anticancer therapy).

**[001757]** Treatment Period (Cohort 3B): up to 12 days, including NMA-LD (7 days), TIL, infusion (1 day) followed by IL-2 administrations (1 to 4 days). The EOT visit occurred

once a patient received the last dose of IL-2. The EOT visit was performed within 30 days after treatment discontinuation and it may be combined with any scheduled visit occurring within this interval during the assessment period.

**[001758]** Assessment Period: began after TIL infusion on Day 0 and ends upon disease progression, with the start of a new anticancer therapy, partial withdrawal of consent to study assessments, or 5 years (Month 60), whichever occurred first. An end-of assessment (EOA) visit occurred once a patient reached disease progression or started a new anticancer therapy.

**[001759]** The TIL autologous therapy with the TILs prepared as described herein was comprised of the following steps:

**[001760]** 1. Tumor resection to provide the autologous tissue that serves as the source of the TIL cellular product;

**[001761]** 2. TIL product produced at a central Good Manufacturing Practice (GMP) facility;

**[001762]** 3. A 7-day NMA-LD preconditioning regimen;

**[001763]** 4. Cohorts 1A, 2A, and 3A: Patients receive a single infusion of pembrolizumab after the completion of their tumor resection for TIL production and baseline scans but before the initiation of NMA-LD regimen. The next dose of pembrolizumab will be no earlier than following the completion of IL-2 and continue Q3W ± 3 days thereafter.

**[001764]** 5. Infusion of the autologous TIL product (Day 0); and

**[001765]** 6. IV IL-2 administrations for up to 6 doses maximum.

**[001766]** In Cohorts 1A, 2A, and 3A, the next dose of pembrolizumab was no earlier than following the completion of IL-2 and continue Q3W ± 3 days thereafter for ≤ 2 years (24 months), or until disease progression or unacceptable toxicity, whichever occurred first.

**[001767]** Flowcharts for Cohorts 1A, 2A, and 3A can be found in Figure 7. The Flowchart for Cohort 3B can be found in Figure 8. Patients were assigned to the appropriate cohort by tumor indication.

**[001768]** <u>TIL Therapy + Pembrolizumab (Cohorts 1A, 2A, and 3A)</u>

**[001769]** Patients were screened and scheduled for surgery for tumor resection. Patients then had one or more tumor lesions resected, which were sent to a central manufacturing facility for TIL production.

**[001770]** Next, the NMA-LD regimen was imitated and consisted of 2 days of IV cyclophosphamide (60 mg/kg) with mesna (per site standard of care or USPI/SmPC) on Days -7 and Day -6 followed by 5 days of IV fludarabine (25 mg/m2: Day -5 through Day -1).

**[001771]** Patients in Cohorts 1A, 2A, and 3A received a single infusion of pembrolizumab after the completion of their tumor resection for TIL production and baseline scans and before the initiation of NMA-LD regimen. IL-2 administrations at a dose of 600,000 IU/kg IV begun as soon as 3 hours after, but no later than 24 hours after, completion of the TIL infusion on Day 0. Additional IL-2 administrations will be given approximately every 8 to 12 hours for up to 6 doses maximum. The second dose of pembrolizumab was no earlier than following the completion of IL-2. Patients should have recovered from all IL-2-related toxicities (Grade $\leq$2), prior to the second pembrolizumab administration. Pembrolizumab will continue Q3W ± 3 days thereafter for $\leq$2 years (24 months) or until disease progression or unacceptable toxicity, whichever occurred first.

**[001772]** TIL Therapy as a Single Agent (Cohort 3B)

**[001773]** Patients were screened and scheduled for surgery for tumor resection. Patients then had one or more tumor lesions resected, which were sent to a central manufacturing facility for TIL production.

**[001774]** Next, the NMA-LD regimen consisted of 2 days of IV cyclophosphamide (60 mg/kg) with mesna (per site standard of care or USPI/SmPC) on Day -7 and Day -6 followed by 5 days of IV fludarabine (25 mg/m2: Day -5 through Day -1).

**[001775]** Infusion of the tumor-derived autologous TIL product occurred no sooner than 24 hours after last dose of fludarabine. IL-2 administrations at a dose of 600,000 IU/kg IV may have begun as soon as 3 hours after, but no later than 24 hours after, completion of the TIL infusion.

**[001776]** Additional IL-2 administrations were given approximately every 8 to 12 hours for up to 6 doses maximum.

**[001777]** Production and Expansion of Tumor Infiltrating Lymphocytes

**[001778]** The TIL autologous cellular product was composed of viable cytotoxic T lymphocytes derived from a patient's tumor/lesion, which are manufactured ex vivo at a central GMP facility. An exemplary flow diagram depicting the TIL production process is provided in Figure 9, for example.

**[001779]** The TIL manufacturing process begun at the clinical site after surgical excision of a primary or secondary metastatic tumor lesion(s) of ≥1.5 cm in diameter in each individual patient. Multiple tumor lesions from various anatomical locations can be excised to compile a total aggregate of tumor tissue; however, the aggregate should not exceed 4.0 cm in diameter, or 10 g in weight, due to the limited quantity of the biopreservation media present in the transport bottle.

**[001780]** Once the tumor lesion(s) was placed in the biopreservation transport bottle, it is shipped at 2°C to 8°C using an express courier to a central GMP manufacturing facility. Upon arrival, the tumor specimen(s) were dissected into fragments, which were then cultured in a pre-rapid expansion protocol (Pre-REP) with human recombinant IL-2 for ~11 days.

**[001781]** These pre-REP cells were then further expanded using a rapid expansion protocol (REP) for 11 days in the presence of IL-2, OKT3 (a murine monoclonal antibody to human CD3, also known as [muromonab-CD3]) and irradiated allogenic peripheral blood mononuclear cells (PBMC) as feeder cells.

**[001782]** The expanded cells were then harvested, washed, formulated, cryopreserved, and shipped to the clinical site via an express courier. The dosage form of the TIL cellular product was a cryopreserved autologous "live-cell suspension" that was ready for infusion into the patient from whom the TIL were derived. Patients were to receive the full dose of product that was manufactured and released, which contained between $1 \times 10^9$ and $150 \times 10^9$ viable cells per the product specification. Clinical experience indicated that objective tumor responses were achieved across this dose range, which has also been shown to be safe (Radvanyi L.G., *et al.*, Clin Cancer Res. **2012**;18(24):6758-70). The full dose of product was provided in up to four infusion bags.

**[001783]** Preparation of Patients to Receive the TIL Cellular Product

**[001784]** The NMA-LD preconditioning regimen used in this study (i.e., 2 days of cyclophosphamide plus mesna, followed by 5 days of fludarabine) was based on the method developed and tested by the National Cancer Institute ( Rosenberg S.A., et al., Clin Cancer Res. **2011**;17(13):4550-7; Radvanyi L.G., et al., Clin Cancer Res. **2012**;18(24):6758-70; Dudley M.E., et al., J Clin Oncol. **2008**;26(32):5233-9; Pilon-Thomas S, et al., J Immunother. **2012**;35(8):615-20; Dudley M.E., et al., J Clin Oncol. **2005**;23(10):2346-57; and Dudley M.E., et al., Science. **2002**;298(5594):850-4). Following the 7-day preconditioning regimen, the patient was infused with the TIL cellular product.

**[001785]** The TIL infusion was followed by the administration of IV IL-2 (600,000 IU/kg) every 8 to 12 hours, with the first dose administered between 3 and 24 hours after the completion of the TIL infusion and continuing for up to 6 doses maximum. Per institutional standards, the doses of IL-2 can be calculated on the basis of actual weight.

**[001786]** SELECTION OF PATIENT POPULATIONS

**[001787]** *Cohort 1A:*

**[001788]** Patients had a confirmed diagnosis of unresectable MM (Stage IIIC or Stage IV, histologically confirmed as per American Joint Committee on Cancer [AJCC] staging system). Ocular melanoma patients were excluded. Patients must not have received prior immuno-oncology targeted agents. If BRAF-mutation positive, patient could have received prior BRAF/MEKtargeted therapy.

**[001789]** *Cohort 2A:*

**[001790]** Patients had advanced, recurrent and/or metastatic HNSCC and can be treatment naive; histologic diagnosis of the primary tumor is required via the pathology report. Patients must not have received prior immunotherapy regimens.

**[001791]** *Cohort 3A:*

**[001792]** Patients had a confirmed diagnosis of Stage III or Stage IV NSCLC (squamous, adenocarcinoma, large cell carcinoma). Patients with oncogene-driven tumors with available effective targeted therapy had received at least one line of targeted therapy.

**[001793]** *Cohort 3B:*

**[001794]** Patients had a confirmed diagnosis of Stage III or Stage IV NSCLC (squamous, adenocarcinoma, large cell carcinoma) and had previously received systemic therapy with CPIs (*e.g.*, anti-PD-1/anti-PD-L1). Patients with oncogene-driven tumors with available effective targeted therapy had received at least one line of targeted therapy.

**[001795]** All patients had received up to 3 prior systemic anticancer therapies (see, inclusion criteria below), excluding immunotherapy for Cohorts 1A, 2A, and 3A. If previously treated, patients had radiographically confirmed progression on or after most recent therapy.

**[001796]** *Inclusion Criteria*

**[001797]** Patients must have met ALL of the following inclusion criteria for participation in the study:

**[001798]** 1. All patients had a histologically or pathologically confirmed diagnosis of malignancy of their respective histologies:

- Unresectable or metastatic melanoma (Cohort 1A)
- Advanced, recurrent or metastatic squamous cell carcinoma of the head and neck (Cohort 2A)
- Stage III or Stage IV NSCLC (squamous, nonsquamous, adenocarcinoma, large cell carcinoma) (Cohorts 3A and 3B).

**[001799]** 2. Cohorts 1A, 2A, and 3A only: Patients were immunotherapy naïve. If previously treated, patients had progressed on or after most recent therapy. Cohorts 1A, 2A, and 3A may have received up to 3 prior systemic anticancer therapies, specifically:

- In Cohort 1A: Patients with unresectable or metastatic melanoma (Stage IIIC or Stage IV); if BRAF mutation-positive, patients could have received a BRAF inhibitor.
- In Cohort 2A: Patients with unresectable or metastatic HNSCC. Those who had received initial chemo-radiotherapy were allowed.
- In Cohort 3A: Patients with Stage III or Stage IV NSCLC (squamous, nonsquamous, adenocarcinoma, or large cell carcinoma) and who were immunotherapy naïve and progressed after ≤3 lines of prior systemic therapy in the locally advanced or metastatic setting. Patients who received systemic therapy in the adjuvant or neoadjuvant setting, or as part of definitive chemoradiotherapy, were eligible and were considered to have had one line of therapy if the disease has progressed within 12 months of completion of prior systemic therapy. Patients with known oncogene drivers (*e.g.*, EGFR, ALK, ROS) who had mutations that were sensitive to targeted therapies must had progressed after at least 1 line of targeted therapy.

**[001800]** 3. Cohort 3B only: Patients with Stage III or Stage IV NSCLC (squamous, nonsquamous, adenocarcinoma, or large cell carcinoma) who had previously received systemic therapy with CPIs (*e.g.*, anti-PD-1/anti-PD-L1) as part of ≤ 3 prior lines of systemic therapy.

- Patients had radiographically confirmed progression on or after most recent therapy.
- Patients who received systemic therapy in the adjuvant or neoadjuvant setting, or as part of definitive chemoradiotherapy, were eligible and were considered to have had 1 line of therapy if the disease had progressed within 12 months of completion of prior systemic therapy.
- Patients with known oncogene drivers (*e.g.*, EGFR, ALK, ROS) who had mutations that are sensitive to targeted therapies must have progressed after at least 1 line of targeted therapy.

**[001801]** 4. Patients had at least 1 resectable lesion (or aggregate lesions) of a minimum 1.5 cm in diameter post-resection for TIL investigational product production. It was encouraged that tumor tissue be obtained from multiple and diverse metastatic lesions, as long as the surgical resection did not pose additional risks to the patient.

- If the lesion considered for resection for TIL generation is within a previously irradiated field, the lesion must have demonstrated radiographic progression prior to resection.
- Patients must have an adequate histopathology specimen for protocol-required testing.

**[001802]** 5. Patients had remaining measurable disease as defined by the standard and well known RECIST 1.1 guidelines (*see*, for example, Eisenhauer, European Journal of Cancer 45:228–247 (2009), also available on the World Wide Web at project.eortc.org/recist/wp-content/uploads/sites/4/2015/03/RECISTGuidelines.pdf ) following tumor resection for TIL manufacturing:

- Lesions in previously irradiated areas were not be selected as target lesions unless there had been demonstrated progression of disease in those lesions;
- Lesions that were partially resected for TIL generation that were still measurable per RECIST may be selected as nontarget lesions but could not serve as a target lesion for response assessment.

**[001803]** 6. Patients were ≥ 18 years at the time of consent.

**[001804]** 7. Patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and an estimated life expectancy of ≥3 months.

**[001805]** 8. Patients of childbearing potential or those with partners of childbearing potential had to be willing to practice an approved method of highly effective birth control during treatment and continue for 12 months after receiving all protocol-related therapy (Note: Females of reproductive potential were to use effective contraception during treatment and for 12 months after their last dose of IL-2, or 4 months after their last dose of pembrolizumab whichever occurred later). Males could not donate sperm during the study or for 12 months after treatment discontinuation, whichever occurred later.

**[001806]** 9. Patients had the following hematologic parameters:

- Absolute neutrophil count (ANC) ≥1000/mm3;
- Hemoglobin ≥9.0 g/dL;
- Platelet count ≥100,000/mm3.

**[001807]** 10. Patients had adequate organ function:

- Serum alanine aminotransferase (ALT)/serum glutamic-pyruvic transaminase (SGPT) and aspartate aminotransferase (AST)/SGOT ≤3 times the upper limit of normal (ULN), patients with liver metastasis ≤5 times ULN.
- An estimated creatinine clearance ≥40 mL/min using the Cockcroft Gault formula at Screening.
- Total bilirubin ≤2 mg/dL.
- Patients with Gilbert's Syndrome must have a total bilirubin ≤3 mg/dL.

**[001808]** 11. Patients were seronegative for the human immunodeficiency virus (HIV1 and HIV2). Patients with positive serology for hepatitis B virus surface antigen (HBsAg), hepatitis B core antibody (anti HBc), or hepatitis C virus (anti-HCV) indicating acute or chronic infection were enrolled depending on the viral load based on polymerase chain reaction (PCR) and the local prevalence of certain viral exposures.

**[001809]** 12. Patients had a washout period from prior anticancer therapy(ies) of a minimum duration, as detailed below prior to the first study treatment (*i.e.*, start of NMA-LD or pembrolizumab):

- Targeted therapy: prior targeted therapy with an epidermal growth factor receptor (EGFR), MEK, BRAF, ALK, ROS1 or other-targeted agents (*e.g.*, erlotinib, afatinib, dacomitinib, osimertinib, crizotinib, ceritinib, lorlatinib)

was allowed provided the washout is a minimum of 14 days prior to the start of treatment.

- Chemotherapy: adjuvant, neoadjuvant or definitive chemotherapy/ chemoradiation was allowed provided the washout is a minimum of 21 days prior to the start of treatment.
- Immunotherapy for Cohort 3B only, prior checkpoint-targeted therapy with an anti-PD-1, other mAbs, or vaccines were allowed with a washout period of ≥ 21 days before the start of NMA-LD.
- Palliative radiation therapy: prior external beam radiation was allowed provided all radiation-related toxicities were resolved to Grade 1 or baseline, excluding alopecia, skin pigmentation change, or other clinically insignificant events, *e.g.*, small area radiation dermatitis or rectal or urinary urgency
- The tumor lesion(s) being assessed as target for response via RECIST 1.1 were outside of the radiation portal; however, if within the portal, they must have demonstrated progression (see Inclusion Criterion above).
- Surgery/pre-planned procedure: previous surgical procedure(s) was permitted provided that wound healing had occurred, all complications had resolved, and at least 14 days have elapsed (for major operative procedures) prior to the tumor resection.

**[001810]** 13. Patients had recovered from all prior anticancer treatment-related adverse events (TRAEs) to Grade ≤1 (per Common Terminology Criteria for Adverse Events [CTCAE]), except for alopecia or vitiligo, prior to cohort assignment.

**[001811]** 14. Patients with stable Grade ≥2 toxicity from prior anticancer therapy were considered on a case by case basis after consultation with the Medical Monitor.

**[001812]** 15. Cohorts 1A, 2A, and 3A patients with irreversible toxicity not reasonably expected to be exacerbated by treatment with pembrolizumab were included only after consultation with the Medical Monitor. For patients in Cohort 3B only, patients with documented Grade ≥2 or higher diarrhea or colitis as a result of a previous treatment with immune checkpoint inhibitor CPI(s) must have been asymptomatic for at least 6 months or had a normal by visual assessment colonoscopy post-treatment prior to tumor resection.

**[001813]** 16. Patients must have provided written authorization for use and disclosure of protected health information.

**[001814]** *Exclusion Criteria*

**[001815]** Patients who meet ANY of the following criteria were excluded from the study:

**[001816]** 1. Patients with melanoma of uveal/ocular origin

**[001817]** 2. Patients who had received an organ allograft or prior cell transfer therapy that included a nonmyeloablative or myeloablative chemotherapy regimen within the past 20 years. (Note: This criterion was applicable for patients undergoing retreatment with TIL, with the exception that they had a prior NMA-LD regimen with their

**[001818]** prior TIL treatment.)

**[001819]** 3. Patients with symptomatic and/or untreated brain metastases.

- o • Patients with definitively-treated brain metastases will be considered for enrollment

**[001820]** after discussion with Medical Monitor; if, prior to the start of treatment the patient is

**[001821]** clinically stable for ≥2 weeks, there are no new brain lesions via magnetic resonance

**[001822]** imaging (MRI) post-treatment, and the patient does not require ongoing

**[001823]** corticosteroid treatment.

**[001824]** 4. Patients who are on a systemic steroid therapy within 21 days of enrollment.

**[001825]** 5. Patients who are pregnant or breastfeeding.

**[001826]** 6. Patients who had an active medical illness(es), which in the opinion of the Investigator, posed increased risks for study participation; such as systemic infections (*e.g.*, syphilis or any other infection requiring antibiotics), coagulation disorders, or other active major medical illnesses of the cardiovascular, respiratory, or immune systems.

**[001827]** 7. Patients may not have active or prior documented autoimmune or inflammatory disorders (including pneumonitis, inflammatory bowel disease [*e.g.*, colitis or Crohn's disease], diverticulitis [with the exception of diverticulosis], systemic lupus

erythematosus, sarcoidosis syndrome, or Wegener syndrome [granulomatosis with polyangiitis, Graves' disease, rheumatoid arthritis, hypophysitis, uveitis, etc.]). The following were exceptions to this criterion:

- Patients with vitiligo or alopecia.
- Patients with hypothyroidism (*e.g.*, following Hashimoto syndrome) stable on
- hormone replacement.
- Any chronic skin condition that did not require systemic therapy.
- Patients with celiac disease controlled by diet alone.

**[001828]** 8. Patients who had received a live or attenuated vaccination within 28 days prior to the start of treatment.

**[001829]** 9. Patients who had any form of primary immunodeficiency (such as severe combined immunodeficiency disease [SCID] and acquired immune deficiency syndrome [AIDS]).

**[001830]** 10. Patients with a history of hypersensitivity to any component of the study drugs. TILs were not administered to patients with a known hypersensitivity to any component of TIL product formulation including, but not limited to any of the following:

- • NMA-LD (cyclophosphamide, mesna, and fludarabine)
- • Proleukin®, aldesleukin, IL-2
- • Antibiotics of the aminoglycoside group (*i.e.*, streptomycin, gentamicin [excluding those who are skin-test negative for gentamicin hypersensitivity])
- • Any component of the TIL product formulation including dimethyl sulfoxide
- [DMSO], HSA, IL-2, and dextran-40
- • Pembrolizumab

**[001831]** 11. Patients who had a left ventricular ejection fraction (LVEF) <45% or who are New York Heart Association Class II or higher. A cardiac stress test demonstrating any irreversible wall movement abnormality in any patients ≥60 years of age or in patients who have a history of ischemic heart disease, chest pain, or clinically significant atrial and/or ventricular arrhythmias.

- Patients with an abnormal cardiac stress test could be enrolled if they had adequate ejection fraction and cardiology clearance with approval of the Sponsor's Medical Monitor.

**[001832]** 12. Patients who had obstructive or restrictive pulmonary disease and have a documented FEV1 (forced expiratory volume in 1 second) of ≤60% of predicted normal.

- • If a patient was not able to perform reliable spirometry due to abnormal upper airway anatomy (*i.e.*, tracheostomy), a 6-minute walk test was used to assess pulmonary function. Patients who were unable to walk a distance of at least 80% predicted for age and sex or demonstrates evidence of hypoxia at any point during the test (SpO2<90%) are excluded.

**[001833]** 13. Patients who had another primary malignancy within the previous 3 years (except for those which did not require treatment or had been curatively treated greater than 1 year ago, and in the judgment of the Investigator, did not pose a significant risk of recurrence including, but not limited to, non-melanoma skin cancer, DCIS, LCIS, prostate cancer Gleason score ≤6 or bladder cancer).

**[001834]** 14. Participation in another clinical study with an investigational product within 21 days of the initiation of treatment.

**[001835]** Study Endpoints and Planned Analyses

**[001836]** The primary and secondary endpoints were analyzed separately by cohort.

**[001837]** *Primary Endpoints:*

**[001838]** The ORR was defined as the proportion of patients who achieved either a confirmed PR or CR as best response as assessed by Investigators per RECIST 1.1 among the efficacy analysis set.

**[001839]** Objective response was evaluated per each disease assessment and the ORR was expressed as a binomial proportion with the corresponding 2-sided 90% CI. The primary analysis for each cohort occurred when all treated patients per cohort have an opportunity to be followed for 12 months, unless progressed/expired or discontinued early from the assessment period.

**[001840]** The safety primary endpoint was measured by any Grade 3 or higher TEAE incidence rate within each cohort expressed as binomial proportions with the corresponding 2-sided 90% CI.

**[001841]** *Secondary Endpoints:*

**[001842]** *Efficacy:*

**[001843]** The secondary efficacy endpoints were defined as follows:

**[001844]** CR rate as based on responders who achieved confirmed CR as assessed by Investigators. DCR was derived as the sum of the number of patients who achieved confirmed PR/CR or sustained SD (at least 6 weeks) divided by the number of patients in the efficacy analysis set × 100%. The CR rate and DCR was summarized using a point estimate and its 2-sided 90% CI.

**[001845]** DOR was defined among patients who achieved objective response. It was measured from the first-time response (PR/CR) criteria are met until the first date that recurrent or progressive disease was objectively documented, or receipt of subsequent anticancer therapy or the patient dies (whichever is first recorded). Patients not experiencing PD or have not died prior to the time of data cut or the final database lock will have their event times censored on the last date that an adequate assessment of tumor status is made.

**[001846]** PFS was defined as the time (in months) from the time of lymphodepletion to PD, or death due to any cause, whichever event is earlier. Patients not experiencing PD or not having expired at the time of the data cut or the final database lock had their event times censored on the last date that an adequate assessment of tumor status is made.

**[001847]** OS was defined as the time (in months) from the time of lymphodepletion to death due to any cause. Patients not having expired by the time of data cut or the final database lock had their event times censored on the last date of their known survival status.

**[001848]** DOR, PFS, and OS was subjected to right censoring. The Kaplan-Meier method will be used to summarize the time-to-event efficacy endpoints. The baseline data for the tumor assessment was the last scan before the lymphodepletion for all cohorts.

**[001849]** The above efficacy parameters will be estimated for applicable cohort for subsets defined by baseline disease characteristics; BRAF status (Cohort 1A only), HPV status (Cohort 2A only), squamous or non-squamous lung disease (Cohorts 3A and 3B only), and anti-PD-L1 status.

## EXAMPLE 11: AN EXEMPLARY EMBODIMENT OF THE GEN 3 EXPANSION PLATFORM AT DAY 0

**[001850]** Prepared tumor wash media. Media warmed prior to start. Added 5 mL of gentamicin (50mg/mL) to the 500 mL bottle of HBSS. Added 5mL of Tumor Wash Media to a 15mL conical to be used for OKT3 dilution. Store at room temperature (RT)

**[001851]** Prepared feeder cell bags. Sterilely transfered feeder cells to feeder cell bags and stored at 37°C until use or freeze. Counted feeder cells if at 37°C. Thawed and then counted feeder cells if frozen.

**[001852]** Optimal range for the feeder cell concentration is between $5x10^4$ and $5x10^6$ cells/mL. Prepared four conical tubes with 4.5mL of AIM-V. Added 0.5 mL of cell fraction for each cell count.

**[001853]** If total viable feeder cell number was $\geq 1 \times 10^9$ cells, proceeded to the next step to adjust the feeder cell concentration. Calculated the volume of feeder cells to remove from the first feeder cell bag in order to add $1 \times 10^9$ cells to a second feeder cell bag.

**[001854]** Using the p1000 micropipette, transferred 900µl of Tumor Wash Media to the OKT3 aliquot (100µL). Using a syringe and sterile technique, drew up 0.6 mL of OKT3 and added into the second feeder cell bag. Adjusted media volume to a total volume of 2L. Transferred the second feeder cells bag to the incubator.

**[001855]** OKT3 formulation details: OKT3 may be aliquoted and frozen in original stock concentration from the vial (1mg/mL) in 100ul aliquots. ~10X aliquots per 1mL vial. Stored at -80C. Day 0: 15ug/flask, *i.e.* 30ng/mL in 500mL – 60ul max ~ 1 aliquot.

**[001856]** Prepared tumor samples. Obtained 6-well plate and 100 mm petri dishes (4 total). Labeled the 6 well plate 'Excess Tumor Pieces'. Labeled one each of the four 100 mm petri dishes as 'Wash_01', 'Wash_02', 'Wash_03', 'Wash_04', and 'Holding'.

**[001857]** Added 5 mL of Tumor Wash Medium into all wells of the 6-well plate labelled Excess Tumor Pieces. Kept the Tumor Wash Medium available for further use in keeping the tumor hydrated during dissection.

**[001858]** Added 50 mL of Tumor Wash Medium to each 100 mm petri dish labelled Wash_01, Wash_02, Wash_03, and Holding. Using a marker, label each petri dish as Dissection 1 through Dissection 4. Incubated the tumor at ambient temperature in Wash_01 for ≥ 3 min. Incubated the tumor at ambient temperature in Wash_02 for ≥ 3 min. Incubated

the tumor at ambient temperature in Wash_03 for ≥ 3 min. After washes were completed, moved tumor to the 'Holding' dish to ensure tissue stays hydrated.

**[001859]** Whitle tumor incubations were in progress, transfered 10 mL of tumor shipping medium into a tube labelled Tumor Shipping Medium. Drew 10mL of the Tumor Shipping Medium into a syringe and inoculated one each anaerobic and aerobic sterility bottle with 5 mL of tumor shipping medium.

**[001860]** Placed ruler under the petri dish lid for the entirety of the dissection process. Measured and recorded length of the tumor and the number of fragments. Dissected the tumor into four intermediate pieces or group into four groups of equivalent volume and conserving the tumor structure of each intermediate piece. Keep tumor pieces hydrated.

**[001861]** Transferred any intermediate tumor pieces not being actively dissected to the Holding dish to keep the tissue hydrated.

**[001862]** Dissected the tumor into 27 $mm^3$ fragments (3x3x3mm), using the ruler under the Dissection dish lid as a reference. Dissected intermediate fragment until 60 fragments were reached. Counted total number of final fragments and prepared G-Rex 100MCS flasks according to the number of final fragments generated (generally 60 fragments per flask).

**[001863]** Retained favorable tissue fragments in the conical tubes labeled as Fragments Tube 1 through Fragments Tube 4. Calculated the number of G-Rex 100MCS flasks to seed with feeder cell suspension according to the number of fragments tubes originated.

**[001864]** Removed feeder cells bag from the incubator and seed the G-Rex 100MCS. Label as D0 (Day 0).

**TUMOR FRAGMENT ADDITION CULTURE IN G-REX100MCS**

**[001865]** Under sterile conditions, unscrewed the cap of the G-Rex 100MCS labelled Tumor Fragments Culture (D0) 1 and the 50 mL conical tube labelled Fragments Tube. Swirled the opened Fragments Tube 1 and, at the same time, slightly lifted the cap of the G-Rex100MCS. Added the medium with the fragments to the G-Rex100MCS while being swirled. Recorded the number of fragments transferred into the G-Rex100MCS.

**[001866]** Once the fragments were located at the bottom of the GREX flask, drew 7 mL of media and created seven 1 mL aliquots – 5 mL for extended characterization and 2 mL for sterility samples. Stored the 5 aliquots (final fragment culture supernatant) for extended characterization at -20°C until needed.

**[001867]** Inoculated one anaerobic BacT/Alert bottle and one aerobic BacT/Alert bottle each with 1mL of final fragment culture supernatant. Repeat for each flask sampled.

## EXAMPLE 12: AN EXEMPLARY EMBODIMENT OF E GEN 3 EXPANSION PLATFORM AT DAY 7-8

**[001868]** Prepared feeder cell bags. Thawed feeder bags for 3-5 minutes in 37°C water bath when frozen. Counted feeder cells if frozen.

**[001869]** Optimal range for the feeder cell concentration is between $5x10^4$ and $5x10^6$ cells/mL. Prepared four conical tubes with 4.5mL of AIM-V. Added 0.5 mL of cell fraction for each cell count into a new cryovial tube. Mixed the samples well and proceeded with the cell count.

**[001870]** If total viable feeder cell number was $\geq 2 x10^9$ cells, proceeded to the next step to adjust the feeder cell concentration. Calculated the volume of feeder cells to remove from the first feeder cell bag in order to add $2 x 10^9$ cells to the second feeder cell bag.

**[001871]** Using the p1000 micropipette, transfer 900µl of HBSS to a 100µL OKT3 aliquot. Mix by pipetting up and down 3 times. Prepared two aliquots.

**[001872]** OKT3 formulation details: OKT3 may be aliquoted and frozen in original stock concentration from the vial (1 mg/mL) in 100ul aliquots. ~10X aliquots per 1mL vial. Stored at -80C. Day7/8: 30ug/flask, *i.e.* 60ng/mL in 500mL – 120ul max ~ 2 aliquots.

**[001873]** Using a syringe and sterile technique, drew up 0.6 mL of OKT3 and added into the feeder cell bag, ensuring all added. Adjusted media volume to a total volume of 2L. Repeated with second OKT3 aliquot and added to the feeder cell bag. Transferred the second feeder cells bag to the incubator.

### PREPARE G-REX100MCS FLASK WITH FEEDER CELL SUSPENSION

**[001874]** Recorded the number of G-Rex 100MCS flasks to process according to the number of G-Rex flasks generated on Day 0. Removed G-Rex flask from incubator and removed second feeder cells bag from incubator.

**Removal of supernatant prior to feeder cell suspension addition:**

**[001875]** Connected one 10 mL syringe to the G-Rex100 flask and drew up 5 mL of media. Created five 1 mL aliquots – 5 mL for extended characterization and storeed the 5

aliquots (final fragment culture supernatant) for extended characterization at -20°C until requested by sponsor. Labeled and repeated for each G-Rex100 flask.

**[001876]** 5-20 X 1 mL samples for characterization, dependeding on number of flasks:

- 5mL = 1flask
- 10mL = 2 flasks
- 15 mL = 3 flasks
- 20 mL =4 flasks

**[001877]** Continued seeding feeder cells into the G-Rex100 MCS and repeated for each G-Rex100 MCS flask. Using sterile transfer methods, gravity transferred 500 mL of the second feeder cells bag by weight (assume 1g = 1mL) into each G-Rex 100MCS flask and recoreded amount. Labeled as Day 7 culture and repeated for each G-Rex100 flask. Transferred G-Rex 100MCS flasks to the incubator.

## EXAMPLE 13: AN EXEMPLARY EMBODIMENT OF THE GEN 3 EXPANSION PLATFORM AT DAY 10-11

**[001878]** Removed the first G-Rex 100MCS flask and using sterile conditions removed 7 mL of Pre-process Culture Supernatant using a 10 mL syringe. Created seven 1 mL aliquots – 5 mL for extended characterization and 2 mL for sterility samples.

**[001879]** Mixed the flask carefully and using a new 10 mL syringe remove 10 mL supernatant and transfer to a 15 mL tube labelled as D10/11 Mycoplasma Supernatant.

**[001880]** Mixed the flask carefully and using a new syringe removed the volume below according to how many flasks were to be processed:

- 1 flask = 40 mL
- 2 flask = 20mL/flask
- 3 flask = 13.3mL/flask
- 4 flask = 10mL/flask

**[001881]** A total of 40 mL should be pulled from all flasks and pooled in a 50mL conical tube labeled 'Day 10/11 QC Sample' and stored in the incubator until needed. Performed a cell count and allocated the cells.

**[001882]** Stored the 5 aliquots (pre-process culture supernatant) for extended characterization at ≤-20°C until needed. Inoculated one anaerobic BacT/Alert bottle and one aerobic BacT/Alert bottle each with 1mL of pre-process culture supernatant.

**[001883]** Continued with cell suspension transferred to the G-Rex 500MCS and repeated for each G-Rex 100MCS. Using sterile conditions, transferred the contents of each G-Rex 100MCS into a G-Rex 500MCS, monitoring about 100 mL of fluid transfer at a time. Stopped transfer when the volume of the G-Rex 100MCS was reduced to 500 mL.

**[001884]** During transfer step, used 10 mL syringe and drew 10 mL of cell suspension into the syringe from the G-Rex 100MCS. Followed the instructions according to the number of flasks in culture. If only 1 flask: Removed 20 mL total using two syringes. If 2 flasks: removed 10 mL per flask. If 3 flasks: removed 7 mL per flask. If 4 flasks: removed 5 mL per flask. Transferred the cell suspension to one common 50 mL conical tube. Keep in the incubator until the cell count step and QC sample. Total number of cells needed for QC was ~ 20e6 cells: 4 x 0.5 mL cell counts (cell counts were undiluted first).

**[001885]** Cells needed for assays:

- 10e6 cells minimum for IFN-G assay
- 1e6 cells for mycoplasma
- 5e6 cells flow CD3+/CD45+

**[001886]** Transferred the G-Rex 500MCS flasks to the incubator.

**Prepared QC samples**

**[001887]** Needed at least 15 x $10^8$. Assays included: Cell count and viability; Mycoplasma (1 x $10^6$ cells/ average viable concentration;) Flow (5 x $10^6$ cells/ average viable concentration;) and IFN-g assay (**5 x $10^6$ cells – 1 x $10^6$ cells**; 8-10 x $10^6$ cells are required for the IFN-γ assay.

**[001888]** Calculated the volume of cells fraction for cryopreservation at 10 x $10^6$ cells/mL and calculated the number of vials to prepare.

## EXAMPLE 14: AN EXEMPLARY EMBODIMENT OF GEN 3 EXPANSION PLATFORM DAY 16-17

Wash Buffer preparation (1% HAS PLASMALYTE A)

**[001889]** Transferred HAS and PLasmalyte to 5L bag to make LOVO wash buffer. Using sterile conditions, transferred a total volume of 125 mL of 25% HSA to the 5L bag. Stored at room temperature.

**[001890]** Removed and transferred 10 mL or 40mL of wash buffer in the 'IL-2 6 x $10^4$ IU/mL' tube (10mL if IL-2 was prepared in advance or 40mL if IL-2 was prepared fresh).

**[001891]** Calculated volume of reconstituted IL-2 to add to Plasmalyte+ 1% HSA: volume of reconstituted IL-2 = (Final concentration of IL-2 x Final volume)/ specific activity of the IL-2 (based on standard assay). The Final Concentration of IL-2 was 6 x $10^4$ IU/mL. The final volume was 40mL.

**[001892]** Removed calculated initial volume of IL-2 needed of reconstituted IL-2 and transfer to the 'IL-2 6x$10^4$ IU/mL' tube. Added 100µL of IL-2 6x$10^6$ IU/mL from the aliquot prepared in advance to the tube labelled 'IL-2 6x$10^4$ IU/mL' containing 10mL of LOVO wash buffer.

**[001893]** Removed about 4500 mL of supernatant from the G-Rex 500MCS flasks. Swirled the remaining supernatant and transferred cells to the Cell Collection Pool bag. Repeated with all G-Rex 500MCS flasks.

**[001894]** Removed 60 mL of supernatant and add to supernatant tubes for quality control assays, including mycoplasma detection. Stored at +2-8°C.

Cell Collection

**[001895]** Counted cells. Prepare four 15mL conicals with 4.5mL of AIM-V. These may be prepared in advance. Optimal range = is between 5x$10^4$ and 5x$10^6$ cells/mL. (1:10 dilution was recommended). For 1:10 dilution, to 4500µL of AIM V prepared previously, add 500µL of CF. Recorded dilution factor.

**[001896]** Calculated the TC (Total Cells) pre-LOVO (live + dead) =

Average Total Cell
Concentration (TC conc pre LOVO)
(live + dead)
X
Volume of Source bag

**[001897]** Calculated the TVC (Total Viable Cells) pre-LOVO (live) =

Average Total Viable Cell
Concentration (TVC pre LOVO)
(live)
X
Volume of LOVO Source Bag

**[001898]** When the total cell (TC) number was > 5 x $10^9$, remove 5 x $10^8$ cells to be cryopreserved as MDA retention samples. 5 x $10^8$ ÷ avg TC concentration (step 14.44) = volume to remove

**[001899]** When the total cell (TC) number was ≤ 5 x $10^9$, remove 4 x $10^6$ cells to be cryopreserved as MDA retention samples. 4 x $10^6$ ÷ avg TC concentration = volume to remove.

**[001900]** Used an appropriately sized syringe to remove the required volume from the LOVO Source Bag. Retained in incubator until cryopreservation steps.

**[001901]** When the total cell number was determined, the number of cells to remove should allow retention of 150x$10^9$ viable cells. Confirm TVC pre-LOVO 5 x $10^8$ or 4 x $10^6$ or not applicable. Calculated the volume of cells to remove.

**[001902]** Calculated the remaining Total Cells Remaining in Bag. Calculated the TC (Total Cells) pre-LOVO. [Avg. Total cell concentration X Remaining Volume = TC pre-LOVO Remaining]

**[001903]** According to the Total number of cells remaining, selected the corresponding process in the following table:

| *Total cells:* | *Retentate (mL)* |
|---|---|
| 0 < Total cells ≤ 31 x $10^9$ | **115** |
| 31 x $10^9$ < Total cells ≤ 71 x $10^9$ | **165** |
| 71 x $10^9$ < Total Cells ≤ 110 x $10^9$ | **215** |
| 110 x $10^9$ < Total Cells≤ 115 x $10^9$ | **265** |

**[001904]** Chose the volume of IL-2 to add corresponding to the used process. Volume calculated as: Retentate Volume x 2 x 300 IU/mL = IU of IL-2 required. IU of IL-2 required / 6 x$10^4$ IU/mL = Volume of IL-2 to add Post LOVO bag. Recorded all volumes added. Obtained samples in cryovial for further analyses.

**[001905]** Mixed the cell product well. Sealed all bags for further processing, included cryopreservation when applicable.

**[001906]** Performed Enodxoton, IFN-γ, sterility, and other assays as needed on cryovial samples obtained.

## EXAMPLE 15: EXEMPLARY GEN 3 PROCESS (ALSO REFERRED TO AS GEN 3.1)

PURPOSE

**[001907]** This example describes further studies regarding the "Comparability between the Gen 2 and Gen 3 processes for TIL expansion". The Gen 3 process was modified to include an activation step early in the process with the goal of increasing the final total viable cell (TVC) output to be comparable (or better) to that in Gen 2, while maintaining the phenotypic and functional profiles as previously seen.

SCOPE

**[001908]** Assessed TVC output through introduction of an activation step to the cultured tumor fragments on Day 0.

**[001909]** Demonstrated comparability in terms of functional and extended phenotypic characterization with the Gen 3 standard, as well as a control arm, across two independent patient tumors.

**[001910]** Analyzed media consumption and metabolite production to confirm processing parameters were maintained at physiologic conditions.

**[001911]** All runs for this example were performed at full-scale platform using commercial donor tumor tissue as the starting material.

INFORMATION

**[001912]** The Process Gen 3 embodiment was modified as a further embodiment and is referred to herein in this example as Gen 3.1.

Gen 3.1 TIL manufacturing concept has four operator interventions:

1. Tumor Fragment Isolation and Activation: On Day 0 of the process the tumor was dissected and the final fragments generated awe~3x3mm each (up to 240 fragments total) and cultured in 1-4 G-Rex100MCS flasks. Each flask contained up to 60 fragments, 500 mL of CM1 or DM1 media, and supplemented with 6,000 IU rhIL-2, 15 μg OKT3, and $2.5x10^8$ irradiated allogeneic mononuclear cells. The culture was incubated at 37°C for 6-8 days.

2. TIL Culture Reactivation: On Day 7-8 the culture was supplemented through slow addition of CM2 or DM1 media supplemented with 6,000 IU rhIL-2, 30 μg OKT3, and $5x10^8$ irradiated allogeneic mononuclear cells in both cases. Care was taken to not disturb the existing cells at the bottom of the flask. The culture was incubated at 37°C for 3-4 days.

3. Culture Scale Up: Occurs on day 10-11. During the culture scale-up, the entire contents of the G-Rex100MCS was transferred to a G-Rex500MCS flask containing 4L of CM4 or DM2 supplemented with 3,000 IU/mL of IL-2 in both cases. Flasks were incubated at 37°C for 5-6 days until harvest.

4. Harvest/Wash/Formulate: On day 16-17 the flasks are volume reduced and pooled. Cells were concentrated and washed with PlasmaLyte A pH 7.4 containing 1% HSA. The washed cell suspension wass formulated at a 1:1 ratio with CryoStor10 and supplemented with rhIL-2 to a final concentration of 300IU/mL.

**[001913]** The DP was cryopreserved with a controlled rate freeze and stored in vapor phase liquid nitrogen. *Complete Standard TIL media 1, 2, or 4 (CM1, CM2, CM4) could be substituted for CTS™OpTmizer™ T-Cell serum free expansion Medium, refered to as Defined Medium (DM1 or DM2), as noted above. For process overview, see for example, Figures 78 and 79.

Process description

**[001914]** On day 0, the tumor was washed 3 times, then fragmented in 3x3x3 final fragments. Once the whole tumor was fragmented, then the final fragments were randomized equally and divided into three pools. One randomized fragment pool was introduced to each arm, adding the same number of fragments per the three experimental matrices.

**[001915]** Tumor L4063 expansion was performed with Standard Media and tumor L4064 expansion was performed with Defined Media (CTS OpTmizer) for the entire TIL expansion process. Components of the media are described herein.

**[001916]** CM1 Complete Media 1: RPMI+ Glutamine supplemented with 2mM Glutamax, 10% Human AB Serum, Gentamicin (50ug/mL), 2-Mercaptoethanol (55uM). Final media formulation supplemented with 6000IU/mL IL-2

**[001917]** CM2 Complete Media 2: 50% CM1 medium + 50% AIM-V medium. Final media formulation supplemented with 6000IU/mL IL-2

**[001918]** CM4 Complete Media 4: AIM-V supplemented with Glutamax (2mM). Final media formulation supplemented with 3000IU/mL IL-2

**[001919]** CTS OpTmizer CTS™OpTmizer™ T-Cell Expansion Basal Medium supplemented with CTS™ OpTmizer™ T-Cell Expansion Supplement (26mL/L).

**[001920]** DM1: CTS™OpTmizer™ T-Cell Expansion Basal Medium supplemented with CTS™ OpTmizer™ T-Cell Expansion Supplement (26mL/L), and CTS™ Immune Cell SR ( 3%), with Glutamax (2mM). Final formulation supplemented with 6,000 IU/mL of IL-2.

**[001921]** DM2: CTS™OpTmizer™ T-Cell Expansion Basal Medium supplemented with CTS™ OpTmizer™ T-Cell Expansion Supplement (26mL/L), and CTS™ Immune Cell SR ( 3%), with Glutamax (2mM). Final formulation supplemented with 3,000 IU/mL of IL-2.

**[001922]** All types of media used, *i.e.*, Complete (CM) and Defined (DM) media, were prepared in advance, held at 4°C degree until the day before use, and warmed at 37°C in an incubator for up to 24 hours in advance prior to process day.

**[001923]** TIL Culture Reactivation occurred on Day 7 for both tumors. Scale-up occurred on day 10 for L4063 and day 11 for L4064. Both cultures were harvested and cryopreserved on Day 16.

Expected Results

**[001924]** Gen 3.1 may reach a higher total viable cells number at harvest on day 16-17 compared to Gen 3.0.

**[001925]** Gen 3.1 may produce similar levels of IFNɣ after restimulation, relative to Gen 3.0.

**[001926]** Gen 3.1 and Gen 3.0 may have a similar clonal diversity, measured by total unique CDR3 sequences present in the final TIL product.

**[001927]** Phenotypic characteristics in the Gen 3.1 process may be similar to Gen 3.0.

RESULTS ACHIEVED

**[001928]** Cells counted and % viability for Gen 3.0 and Gen 3.1 processes were determined. Expansion in all the conditions followed details described in this example.

Total viable cell counts and fold expansion

**[001929]** For each tumor, the fragments were divided into three pools of equal numbers. Due to the small size of the tumors, the maximum number of fragments per flask was not achieved. For the three different processes, the total viable cells and cell viability were assessed for each condition. Cell counts were determined as TVC on day 7 for reactivation, TVC on day 10 (L4064) or day 11 (L4063) for scale-up, and TVC at harvest on day 16/17.

**[001930]** Cell counts for Day 7 and Day 10/11 were taken FIO. Fold expansion was calculated by dividing the harvest day 16/17 TVC by the day 7 reactivation day TVC. To compare the three arms, the TVC on harvest day was divided by the number of fragments added in the culture on Day 0 in order to calculate an average of viable cells per fragment.

**[001931]** Cell counts and viability assays were performed for L4063 and L4064. As indicated in Figure 89, the Gen 3.1-Test process yielded more cells per fragment than the Gen 3.0 Process on both tumors.

**[001932]** Figure 90: Total viable cell count and fold expansion

% Viability during the process

**[001933]** On reactivation, scale up and harvest the percent viability was performed on all conditions. On day 16/17 harvest, the final TVC were compared against release criteria for % viability. All of the conditions assessed surpassed the 70% viability criterion and were comparable across processes and tumors as is shown in Figure 82.

Immunophenotyping

**[001934]** Phenotypic characterization on TIL final product.

**[001935]** The final products were subjected to flow cytometry analysis to test purity, differentiation, and memory markers. Percent populations were consistent for TCRα/β, CD4+ and CD8+ cells for all conditions.

**[001936]** Extended phenotypic analysis of REP TIL was performed. TIL product showed a higher percentage of CD4+ cells for Gen 3.1 conditions compared to Gen 3.0 on both tumors, and higher percentage of CD28+ cells from CD8+ population for Gen 3.0 compared to Gen 3.1 conditions on both conditions.

**[001937]** TIL harvested from the Gen 3.0 and Gen 3.1 processes showed comparable phenotypic markers as CD27 and CD56 expression on CD4+and CD8+ cells, and a comparable CD28 expression on CD4+ gated cells population. Figure 83 shows the

phenotypic markers of the final TIL product for Gen 3.0 and Gen 3.1 processes. Figure 83: Phenotypic characterization of final TIL product for L4063 and L4064.

Memory markers comparison on TIL final product:

**[001938]** Figure 84 shows the memory markers of TIL final product that were determined by multicolor flow cytometry. Frozen samples of TIL harvested on day 16 were stained for analysis. TIL memory status was comparable between Gen 3.0 and Gen 3.1 processes. Figure 84: Memory marker analysis of TIL product for L4063 and L4064.

Activation and exhaustion markers comparison on TIL final product:

**[001939]** Figures 85 and 86 show the activation and exhaustion of TIL final product that were determined by multicolor flow cytometry. Activation and exhaustion markers were comparable between the Gen 3.0 and Gen 3.1 processes gated on CD4+ (Figure 85) and CD8+ (Figure 86) cells. Figure 85: Activation and exhaustion markers gated on CD4+ cells for L4063 and L4064. Figure 86: Activation and exhaustion markers gated on CD8+ cells for L4063 and L4064.

Interferon gamma secretion upon restimulation:

**[001940]** Harvested TIL underwent an overnight restimulation with coated anti-CD3 plates for L4063 and L4064. Higher production of IFNγ from the Gen 3.1 process was observed in the two tumors analyzed compared to Gen 3.0 process. The IFNγ production on each condition is shown in Figure 87. Figure 87: IFN-γ production (pg/mL) for final product on processes Gen 3.0 and Gen 3.1.

Measurement of IL-2 levels in culture media

**[001941]** To compare the levels of IL-2 consumption between all of the conditions and processes, cell supernatants were collected at initiation of reactivation on Day 7, at scale-up Day 10 (L4064) / 11 (L4063), and at harvest Day 16 / 17, and frozen. The supernatants were subsequently thawed and then analyzed. The quantity of IL-2 in cell culture supernatant was measured by the manufacturer protocol. Data are shown in Figure 88.

**[001942]** Overall Gen 3 and Gen 3.1 processes were comparable in terms of IL-2 consumption during the complete process assessed across same media conditions. Figure 88: IL-2 concentration (pg/mL) analysis on spent media collected for L4063 and L4064.

Metabolite analysis

**[001943]** Spent media supernatants was collected from L4063 and L4064 at reactivation initiation on day 7, scale-up on day 10 (L4064) or day 11 (L4063), and at harvest on days 16/17 for L4063 and L4064, for every condition. Supernatants were analyzed on a CEDEX Bio-analyzer for concentrations of glucose, lactate, glutamine, glutamax, and ammonia. Figure 89 shows the concentration of glucose in spent media collected for Gen 3.0 and Gen 3.1 processes for L4063 and L4064 tumors.

**[001944]** Defined media has a higher glucose concentration of 4.5 g/L compared to complete media (2g/L). Overall, the concentration and consumption of glucose were comparable for Gen 3.0 and Gen 3.1 processes within each media type. Figure 89: Concentration of glucose (g/L) in spent media for L4063 and L4064.

**[001945]** An increase in lactate was observed for both tumors, L4063 and L4064, for all test conditions. The increase in lactate was comparable between the Gen 3.0 and Gen 3.1 conditions and between the two media used for reactivation expansion (complete media for L4063 and defined media for L4064).

**[001946]** Figure 90 shows the concentration of lactate on spent media collected for Gen 3.0 and Gen 3.1 processes conditions for both tumors L4063 and L4064. Figure 90: Concentration of lactate (g/L) in spent media for L4063 and L4064.

**[001947]** In the case of L4063, the standard basal media contained 2 mM L-glutamine and was supplemented with 2mM glutamax to compensate for the natural degradation of L-glutamine in culture conditions to L-glutamate and ammonia.

**[001948]** For L4064 tumor, defined (serum free) media used did not contain L-glutamine on the basal media, and was supplemented only with glutamax to a final concentration of 2mM. Glutamax is a dipeptide of L-alanine and L-glutamine, is more stable then L-glutamine in aqueous solutions and does not spontaneously degrade into glutamate and and ammonia. Instead, the dipeptide is gradually dissociated into the individual amino acids, thereby maintaining a lower but sufficient concentration of L-glutamine to sustain robust cell growth. Figure 91 and Figure 92 shows the concentration of glutamine and glutamax respectively on spent media collected on Gen 3.0 and Gen 3.1 processes conditions for both tumors L4063 and L4064.

**[001949]** For L4063, the concentration of glutamine and glutamax slightly decreased on the scale-up day, but at harvest day showed an increase to similar or closer levels compared to reactivation day. For L4064, glutamine and glutamax concentration showed a slight

degradation in a similar rate between different conditions, during the whole process. Figure 91: Concentration of glutamine (mmol/L) in spent media for L4063 and L4064. Figure 92: Concentration of glutamax (mmol/L) in spent media for L4063 and L4064.

**[001950]** Figure 93 shows the concentration of ammonia on spent media collected for Gen 3.0 and Gen 3.1 processes for both tumors L4063 and L4064. As expected, ammonia concentrations were higher for L4063 (grown in standard media containing 2 mM glutamine + 2 mM glutamax) than L4064 (grown in defined media containing 2 mM glutamax). Further, as expected, there was a gradual increase or accumulation of ammonia over the course of the culture. There were no differences in ammonia concentrations across the three different test conditions. Figure 93: Concentration of Ammonia (mmol/L) in spent media for L4063 and L4064.

Telomere repeats by Flow – FISH:

**[001951]** Flow-FISH technology was used to measure the average length of the telomere repeat on L4063 and L4064 under Gen 3 and Gen 3.1 processes. The determination of a relative telomere length (RTL) was calculated using Telomere PNA kit/FITC for flow cytometry analysis from DAKO. Telomere assay was performed.

**[001952]** Telomere length in samples of L4063 an L4064, were compared to a control cell line (1301 leukemia). The control cell line is a tetraploid cell line having long stable telomeres, that allows calculation of a relative telomere length. Gen 3 and Gen 3.1 processes assessed in both tumors showed comparable telomere length as shown in Figure 94. Figure 94: Summary of Relative Telomere Length (RTL) Compared to the Control (1301) Cell Line.

TCR Vβ repertoire Analysis

**[001953]** To determine the clonal diversity of the cell products generated in each process, TIL final products were assayed for clonal diversity analysis through sequencing of the CDR3 portion of the T-cell receptors.

**[001954]** Three parameters were compared between the three conditions:

- Diversity index of Unique CDR3 (uCDR3)
- % shared uCDR3
- For the top 80% of uCDR3:
    - Compare the % shared uCDR3 copies

- Compare the frequency of unique clonotypes

**[001955]** Figure 95: TCR Vβ repertoire summary for L4063 and L4064. Describes the clonality of TIL for final TIL product on tumor L4063 and L4064 on Gen 3 and Gen 3.1 conditions as measured by the TCR Vβ repertoire.

**[001956]** Figure 97: Comparison between Gen 3 and Gen3.1 Control and Gen 3.1 Test, percentage shared unique CDR3 sequences on L4063 on TIL harvested cell product for: 975 sequences are shared between Gen 3 and Gen 3.1 Test final product, equivalent to 88% of top 80% of unique CDR3 sequences from Gen 3 shared with Gen 3.1 Test final product.

**[001957]** Figure 96: L4063 Frequency comparison of unique CDR3 sequences on TIL harvested final cell product between Gen 3.0 and Gen 3.1 processes.

**[001958]** Figure 98: Comparison between Gen 3 and Gen3.1 Control and Gen 3.1 Test, percentage shared unique CDR3 sequences on L4064 on TIL harvested cell product for: 2163 sequences are shared between Gen 3 and Gen 3.1 Test final product, equivalent to 87% of top 80% of unique CDR3 sequences from Gen 3 shared with Gen 3.1 Test final product.

**[001959]** Figure 99: L4064 Frequency comparison of unique CDR3 sequences on TIL harvested final cell product between Gen 3.0 and Gen 3.1 processes.

**[001960]** The number of unique CD3 sequences identified from $1x10^6$ cells collected on Harvest day 16, for the different processes. Gen 3.1 Test condition showed a slightly higher clonal diversity compared to Gen 3.0 based on the number of unique peptide CDRs within the sample.

**[001961]** Shanon entropy diversity index is a more reliable and common metric for comparison, for Gen 3.1 conditions on both tumors showed slightly higher diversity than Gen 3 process, suggesting that TCR Vβ repertoire for Gen 3.1 Test condition is more polyclonal than the Gen 3.0 process.

**[001962]** Additionally, the TCR Vβ repertoire for Gen 3.1 Test condition showed more than 87% overlap with the corresponding repertoire for Gen 3.0 process on both tumor L4063 and L4064.

ADDITIONAL INFORMATION

**[001963]** The value of IL-2 concentration on spent media for Gen 3.1 Test L4064 on reactivation day was below to the expected value (similar to Gen 3.1 control and Gen 3.0 condition).

**[001964]** The low value could be due to a pipetting error, but because of the minimal sample taken it was not possible to repeat the assay.

**[001965]** Spent media from scale up day 10/ 11 on sample L4064 was not collected, and not included in the analysis of IL-2 concentration and metabolite analysis on supernatant.

CONCLUSIONS

**[001966]** Gen 3.1 test condition including feeders and OKT-3 on Day 0 showed a higher TVC of cell doses at Harvest day 16 compared to Gen 3.0 and Gen 3.1 control. TVC on the final product for Gen 3.1 test condition was around 2.5 times higher than Gen 3.0.

**[001967]** Gen 3.1 test condition with the addition of OKT-3 and feeders on day 0, for both tumors L4063 and L4064, reached a maximum capacity of the flask at harvest. Under these conditions, if a maximum of 4 flasks on day 0 is initiated, the final cell dose could be between 80 - 100E+09 TILs.

**[001968]** All the quality attributes such as phenotypic characterization including purity, exhaustion, activation and memory markers on final TIL product were maintained and comparable between Gen 3.1 Test and Gen 3.0 process. Telomere length on TIL final product and IL-2 consumption on spent media were comparable between Gen 3.0 and Gen 3.1 processes.

**[001969]** IFN gamma production on final TIL product was 3 times higher on Gen 3.1 with feeder and OKT-3 addition on day 0, compared to Gen 3.0 in the two tumors analyzed, suggesting Gen 3.1 process generated a potent TIL product.

**[001970]** No differences observed in glucose or lactate levels across test conditions. No differences observed on glutamine and ammonia between Gen 3.0 and Gen 3.1 processes across media conditions. The low levels of glutamine on the media are not limiting cell growth and suggest the addition of glutamax only in media is sufficient to give the nutrients needed to make cells proliferate.

**[001971]** The scale up day for L4063 and L4064 was on day 11 and day 10 respectively and did not show major differences in terms of cell number reached on the harvest day of the process and metabolite consumption was comparable in both cases during the whole process.

This observation suggests of Gen 3.0 optimized process can have flexibility on processing days, thereby facilitating flexibility in the manufacturing schedule.

**[001972]** Gen 3.1 process with feeder and OKT-3 addition on day 0 showed a higher clonal diversity measured by CDR3 TCRab sequence analysis compared to Gen 3.0.

**[001973]** Figure 100 describes an embodiment of the Gen 3 process (Gen 3 Optimized process). Standard media and CTS Optimizer serum free media can be used for Gen 3 Optimized process TIL expansion. In case of CTS Optimizer serum free media is recommended to increase the glutamax on the media to final concentration 4mM.

**Feasibility and Comparability:**

Feasibility:

**[001974]** Feasibility was established for all study conditions in all experiments. Across all the experiments and conditions and between the donor tumor tissue, all the experiments were performed utilizing the same lots of critical raw material such as IL-2, Human Serum-AB, allogeneic feeder cells, OKT-3.

Comparability:

**[001975]** Comparability was determined by the ability of any arm of the study to meet release criteria of our clinical product according to LFP-002 Autologous Tumor infiltrating Lymphocytes (TIL) cryopreserved Day 22, as outlined in Figure 101.

## EXAMPLE 16: TUMOR EXPANSION PROCESSES WITH DEFINED MEDIUM.

**[0048]** The processes disclosed in Examples 7 through 15 are performed with substituting the CM1 and CM2 media with a defined medium according to the present invention (*e.g.*, CTS™ OpTmizer™ T-Cell Expansion SFM, ThermoFisher, including for example DM1 and DM2).

## EXAMPLE 17: A PHASE 2, MULTICENTER STUDY OF AUTOLOGOUS TUMOR INFILTRATING LYMPHOCYTES IN PATIENTS WITH SOLID TUMORS

**STUDY DESIGN**

**[001976]** Overview

**[001977]** This example describes a prospective, open-label, multi-cohort, non-randomized, multicenter Phase 2 study evaluating ACT using TIL in combination with pembrolizumab or TIL as a single therapy, using TILs prepared as described in the present application as well as in this example.

**[001978]** Objectives:

**[001979]** *Primary:*

**[001980]** To evaluate the efficacy of autologous TIL in combination with pembrolizumab in MM, HNSCC, or NSCLC patients or TIL as a single therapy in relapsed or refractory (r/r) NSCLC patients, who had previously progressed on or after treatment with CPIs, as determined by objective response rate (ORR), using the Response Evaluation Criteria in Solid Tumors (RECIST 1.1), as assessed by Investigator.

**[001981]** To characterize the safety profile of TIL in combination with pembrolizumab in MM, HNSCC, and NSCLC patients or TIL as a single therapy in r/r NSCLC patients as measured by the incidence of Grade $\geq$ 3 treatment-emergent adverse events (TEAEs).

**[001982]** *Secondary:*

**[001983]** To further evaluate the efficacy of autologous TIL in combination with pembrolizumab in MM, HNSCC, and NSCLC patients or TIL as a single therapy in r/r NSCLC patients using complete response (CR) rate, duration of response (DOR), disease control rate (DCR), progression-free survival (PFS) using RECIST 1.1, as assessed by Investigator, and overall survival (OS).

**[001984]** Cohorts:

**[001985]** Cohort 1A: TIL therapy in combination with pembrolizumab in patients with Stage IIIC or Stage IV unresectable or MM with $\leq$ 3 prior lines of systemic therapy excluding immunotherapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001986]** Cohort 2A: TIL therapy in combination with pembrolizumab in patients with advanced, recurrent or metastatic HNSCC (*e.g.*, Stages T1N1-N2B, T2-4N0-N2b) with $\leq$ 3 prior lines of systemic therapy, excluding immunotherapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001987]** Cohort 3A: TIL therapy in combination with pembrolizumab in patients with locally advanced or metastatic (Stage III– IV) NSCLC with $\leq$3 prior lines of systemic

therapy, excluding immunotherapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001988]** Cohort 3B: TIL therapy as a single agent in patients Stage III or Stage IV NSCLC who have previously received systemic therapy with CPIs (*e.g.*, anti-PD-1/anti-PD-L1) as part of ≤ 3 prior lines of systemic therapy. If previously treated, patients must have had radiographically documented progression on or after most recent therapy.

**[001989]** Patients in Cohorts 3A and 3B (NSCLC) with oncogene-driven tumors with available effective targeted therapy must have received at least one line of targeted therapy.

**[001990]** All patients received autologous cryopreserved TIL therapy (with or without pembrolizumab, depending on cohort assignment), preceded by a nonmyeloablative lymphodepletion (NMA-LD) preconditioning regimen consisting of cyclophosphamide and fludarabine. Following TIL infusion, up to 6 IV interleukin-2 (IL-2) doses maximum were administered.

**[001991]** The following general study periods took place in all 4 cohorts, unless specified otherwise.

**[001992]** Screening and Tumor Resection: Up to 4 weeks (28 days) from study entry; manufacturing of the TIL Product: approximately ≤22 days from tumor resection; and treatment period, as discussed below.

**[001993]** Treatment Period (Cohorts 1A, 2A, and 3A): up to 2 years, including NMA-LD (7 days), TIL infusion (1 day) followed by IL-2 administrations (1 to 4 days).Patients receive a single infusion of pembrolizumab after the completion of their tumor resection for TIL production and baseline scans but before the initiation of the NMA-LD regimen. The next dose of pembrolizumab will be no earlier than following the completion of IL-2 and continue Q3W ± 3 days thereafter for ≤ 2 years (24 months) or until disease progression or unacceptable toxicity, whichever occurs first. The end-of-treatment (EOT) visit occurred within 30 days after the last dose of pembrolizumab. The visit could be combined with end-of-assessment (EOA) visit if applicable (*e.g.*, pembrolizumab discontinuation occurred at disease progression or at the start of new anticancer therapy).

**[001994]** Treatment Period (Cohort 3B): up to 12 days, including NMA-LD (7 days), TIL, infusion (1 day) followed by IL-2 administrations (1 to 4 days). The EOT visit occurred once a patient received the last dose of IL-2. The EOT visit was performed within 30 days

after treatment discontinuation and it may be combined with any scheduled visit occurring within this interval during the assessment period.

**[001995]** Assessment Period: began after TIL infusion on Day 0 and ends upon disease progression, with the start of a new anticancer therapy, partial withdrawal of consent to study assessments, or 5 years (Month 60), whichever occurred first. An end-of assessment (EOA) visit occurred once a patient reached disease progression or started a new anticancer therapy.

**[001996]** The TIL autologous therapy with the TILs prepared as described herein was comprised of the following steps:

**[001997]** 1. Tumor resection to provide the autologous tissue that serves as the source of the TIL cellular product;

**[001998]** 2. TIL product produced at a central Good Manufacturing Practice (GMP) facility;

**[001999]** 3. A 7-day NMA-LD preconditioning regimen;

**[002000]** 4. Cohorts 1A, 2A, and 3A: Patients receive a single infusion of pembrolizumab after the completion of their tumor resection for TIL production and baseline scans but before the initiation of NMA-LD regimen. The next dose of pembrolizumab will be no earlier than following the completion of IL-2 and continue Q3W ± 3 days thereafter.

**[002001]** 5. Infusion of the autologous TIL product (Day 0); and

**[002002]** 6. IV IL-2 administrations for up to 6 doses maximum.

**[002003]** In Cohorts 1A, 2A, and 3A, the next dose of pembrolizumab was no earlier than following the completion of IL-2 and continue Q3W ± 3 days thereafter for ≤ 2 years (24 months), or until disease progression or unacceptable toxicity, whichever occurred first.

**[002004]** Flowcharts for Cohorts 1A, 2A, and 3A can be found in Figure 7. The Flowchart for Cohort 3B can be found in Figure 8. Patients were assigned to the appropriate cohort by tumor indication.

**[002005]** <u>TIL Therapy + Pembrolizumab (Cohorts 1A, 2A, and 3A)</u>

**[002006]** Patients were screened and scheduled for surgery for tumor resection. Patients then had one or more tumor lesions resected, which were sent to a central manufacturing facility for TIL production.

**[002007]** Next, the NMA-LD regimen was imitated and consisted of 2 days of IV cyclophosphamide (60 mg/kg) with mesna (per site standard of care or USPI/SmPC) on Days -7 and Day -6 followed by 5 days of IV fludarabine (25 mg/m2: Day -5 through Day -1).

**[002008]** Patients in Cohorts 1A, 2A, and 3A received a single infusion of pembrolizumab after the completion of their tumor resection for TIL production and baseline scans and before the initiation of NMA-LD regimen. IL-2 administrations at a dose of 600,000 IU/kg IV begun as soon as 3 hours after, but no later than 24 hours after, completion of the TIL infusion on Day 0. Additional IL-2 administrations will be given approximately every 8 to 12 hours for up to 6 doses maximum. The second dose of pembrolizumab was no earlier than following the completion of IL-2. Patients should have recovered from all IL-2-related toxicities (Grade ≤2), prior to the second pembrolizumab administration. Pembrolizumab will continue Q3W ± 3 days thereafter for ≤2 years (24 months) or until disease progression or unacceptable toxicity, whichever occurred first.

**[002009]** TIL Therapy as a Single Agent (Cohort 3B)

**[002010]** Patients were screened and scheduled for surgery for tumor resection. Patients then had one or more tumor lesions resected, which were sent to a central manufacturing facility for TIL production.

**[002011]** Next, the NMA-LD regimen consisted of 2 days of IV cyclophosphamide (60 mg/kg) with mesna (per site standard of care or USPI/SmPC) on Day -7 and Day -6 followed by 5 days of IV fludarabine (25 mg/m2: Day -5 through Day -1).

**[002012]** Infusion of the tumor-derived autologous TIL product occurred no sooner than 24 hours after last dose of fludarabine. IL-2 administrations at a dose of 600,000 IU/kg IV may have begun as soon as 3 hours after, but no later than 24 hours after, completion of the TIL infusion.

**[002013]** Additional IL-2 administrations were given approximately every 8 to 12 hours for up to 6 doses maximum.

**[002014]** Production and Expansion of Tumor Infiltrating Lymphocytes

**[002015]** The TIL autologous cellular product was composed of viable cytotoxic T lymphocytes derived from a patient's tumor/lesion, which are manufactured ex vivo at a central GMP facility. An exemplary flow diagram depicting the TIL production process is provided in Figure 9, for example.

**[002016]** The TIL manufacturing process begun at the clinical site after surgical excision of a primary or secondary metastatic tumor lesion(s) of ≥1.5 cm in diameter in each individual patient. Multiple tumor lesions from various anatomical locations can be excised to compile a total aggregate of tumor tissue; however, the aggregate should not exceed 4.0 cm in diameter, or 10 g in weight, due to the limited quantity of the biopreservation media present in the transport bottle.

**[002017]** Once the tumor lesion(s) was placed in the biopreservation transport bottle, it is shipped at 2°C to 8°C using an express courier to a central GMP manufacturing facility. Upon arrival, the tumor specimen(s) were dissected into fragments, which were then cultured in a pre-rapid expansion protocol (Pre-REP) with human recombinant IL-2 for ~11 days.

**[002018]** These pre-REP cells were then further expanded using a rapid expansion protocol (REP) for 11 days in the presence of IL-2, OKT3 (a murine monoclonal antibody to human CD3, also known as [muromonab-CD3]) and irradiated allogenic peripheral blood mononuclear cells (PBMC) as feeder cells.

**[002019]** The expanded cells were then harvested, washed, formulated, cryopreserved, and shipped to the clinical site via an express courier. The dosage form of the TIL cellular product was a cryopreserved autologous "live-cell suspension" that was ready for infusion into the patient from whom the TIL were derived. Patients were to receive the full dose of product that was manufactured and released, which contained between $1 \times 10^9$ and $150 \times 10^9$ viable cells per the product specification. Clinical experience indicated that objective tumor responses were achieved across this dose range, which has also been shown to be safe (Radvanyi L.G., *et al.*, Clin Cancer Res. **2012**;18(24):6758-70). The full dose of product was provided in up to four infusion bags.

**[002020]** Preparation of Patients to Receive the TIL Cellular Product

**[002021]** The NMA-LD preconditioning regimen used in this study (i.e., 2 days of cyclophosphamide plus mesna, followed by 5 days of fludarabine) was based on the method developed and tested by the National Cancer Institute ( Rosenberg S.A., et al., Clin Cancer Res. **2011**;17(13):4550-7; Radvanyi L.G., et al., Clin Cancer Res. **2012**;18(24):6758-70; Dudley M.E., et al., J Clin Oncol. **2008**;26(32):5233-9; Pilon-Thomas S, et al., J Immunother. **2012**;35(8):615-20; Dudley M.E., et al., J Clin Oncol. **2005**;23(10):2346-57; and Dudley M.E., et al., Science. **2002**;298(5594):850-4). Following the 7-day preconditioning regimen, the patient was infused with the TIL cellular product.

**[002022]** The TIL infusion was followed by the administration of IV IL-2 (600,000 IU/kg) every 8 to 12 hours, with the first dose administered between 3 and 24 hours after the completion of the TIL infusion and continuing for up to 6 doses maximum. Per institutional standards, the doses of IL-2 can be calculated on the basis of actual weight.

**[002023]** SELECTION OF PATIENT POPULATIONS

**[002024]** *Cohort 1A:*

**[002025]** Patients had a confirmed diagnosis of unresectable MM (Stage IIIC or Stage IV, histologically confirmed as per American Joint Committee on Cancer [AJCC] staging system). Ocular melanoma patients were excluded. Patients must not have received prior immuno-oncology targeted agents. If BRAF-mutation positive, patient could have received prior BRAF/MEKtargeted therapy.

**[002026]** *Cohort 2A:*

**[002027]** Patients had advanced, recurrent and/or metastatic HNSCC and can be treatment naive; histologic diagnosis of the primary tumor is required via the pathology report. Patients must not have received prior immunotherapy regimens.

**[002028]** *Cohort 3A:*

**[002029]** Patients had a confirmed diagnosis of Stage III or Stage IV NSCLC (squamous, adenocarcinoma, large cell carcinoma). Patients with oncogene-driven tumors with available effective targeted therapy had received at least one line of targeted therapy.

**[002030]** *Cohort 3B:*

**[002031]** Patients had a confirmed diagnosis of Stage III or Stage IV NSCLC (squamous, adenocarcinoma, large cell carcinoma) and had previously received systemic therapy with CPIs (*e.g.*, anti-PD-1/anti-PD-L1). Patients with oncogene-driven tumors with available effective targeted therapy had received at least one line of targeted therapy.

**[002032]** All patients had received up to 3 prior systemic anticancer therapies (see, inclusion criteria below), excluding immunotherapy for Cohorts 1A, 2A, and 3A. If previously treated, patients had radiographically confirmed progression on or after most recent therapy.

**[002033]** *Inclusion Criteria*

**[002034]** Patients must have met ALL of the following inclusion criteria for participation in the study:

**[002035]** 1. All patients had a histologically or pathologically confirmed diagnosis of malignancy of their respective histologies:

- Unresectable or metastatic melanoma (Cohort 1A)
- Advanced, recurrent or metastatic squamous cell carcinoma of the head and neck (Cohort 2A)
- Stage III or Stage IV NSCLC (squamous, nonsquamous, adenocarcinoma, large cell carcinoma) (Cohorts 3A and 3B).

**[002036]** 2. Cohorts 1A, 2A, and 3A only: Patients were immunotherapy naïve. If previously treated, patients had progressed on or after most recent therapy. Cohorts 1A, 2A, and 3A may have received up to 3 prior systemic anticancer therapies, specifically:

- In Cohort 1A: Patients with unresectable or metastatic melanoma (Stage IIIC or Stage IV); if BRAF mutation-positive, patients could have received a BRAF inhibitor.
- In Cohort 2A: Patients with unresectable or metastatic HNSCC. Those who had received initial chemo-radiotherapy were allowed.
- In Cohort 3A: Patients with Stage III or Stage IV NSCLC (squamous, nonsquamous, adenocarcinoma, or large cell carcinoma) and who were immunotherapy naïve and progressed after ≤3 lines of prior systemic therapy in the locally advanced or metastatic setting. Patients who received systemic therapy in the adjuvant or neoadjuvant setting, or as part of definitive chemoradiotherapy, were eligible and were considered to have had one line of therapy if the disease has progressed within 12 months of completion of prior systemic therapy. Patients with known oncogene drivers (*e.g.*, EGFR, ALK, ROS) who had mutations that were sensitive to targeted therapies must had progressed after at least 1 line of targeted therapy.

**[002037]** 3. Cohort 3B only: Patients with Stage III or Stage IV NSCLC (squamous, nonsquamous, adenocarcinoma, or large cell carcinoma) who had previously received systemic therapy with CPIs (*e.g.*, anti-PD-1/anti-PD-L1) as part of ≤ 3 prior lines of systemic therapy.

- Patients had radiographically confirmed progression on or after most recent therapy.
- Patients who received systemic therapy in the adjuvant or neoadjuvant setting, or as part of definitive chemoradiotherapy, were eligible and were considered to have had 1 line of therapy if the disease had progressed within 12 months of completion of prior systemic therapy.
- Patients with known oncogene drivers (*e.g.*, EGFR, ALK, ROS) who had mutations that are sensitive to targeted therapies must have progressed after at least 1 line of targeted therapy.

**[002038]** 4. Patients had at least 1 resectable lesion (or aggregate lesions) of a minimum 1.5 cm in diameter post-resection for TIL investigational product production. It was encouraged that tumor tissue be obtained from multiple and diverse metastatic lesions, as long as the surgical resection did not pose additional risks to the patient.

- If the lesion considered for resection for TIL generation is within a previously irradiated field, the lesion must have demonstrated radiographic progression prior to resection.
- Patients must have an adequate histopathology specimen for protocol-required testing.

**[002039]** 5. Patients had remaining measurable disease as defined by the standard and well known RECIST 1.1 guidelines (*see*, for example, Eisenhauer, European Journal of Cancer 45:228–247 (2009), also available on the World Wide Web at project.eortc.org/recist/wp-content/uploads/sites/4/2015/03/RECISTGuidelines.pdf ) following tumor resection for TIL manufacturing:

- Lesions in previously irradiated areas were not be selected as target lesions unless there had been demonstrated progression of disease in those lesions;
- Lesions that were partially resected for TIL generation that were still measurable per RECIST may be selected as nontarget lesions but could not serve as a target lesion for response assessment.

**[002040]** 6. Patients were ≥ 18 years at the time of consent.

**[002041]** 7. Patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and an estimated life expectancy of ≥3 months.

**[002042]** 8. Patients of childbearing potential or those with partners of childbearing potential had to be willing to practice an approved method of highly effective birth control during treatment and continue for 12 months after receiving all protocol-related therapy (Note: Females of reproductive potential were to use effective contraception during treatment and for 12 months after their last dose of IL-2, or 4 months after their last dose of pembrolizumab whichever occurred later). Males could not donate sperm during the study or for 12 months after treatment discontinuation, whichever occurred later.

**[002043]** 9. Patients had the following hematologic parameters:

- Absolute neutrophil count (ANC) ≥1000/mm3;
- Hemoglobin ≥9.0 g/dL;
- Platelet count ≥100,000/mm3.

**[002044]** 10. Patients had adequate organ function:

- Serum alanine aminotransferase (ALT)/serum glutamic-pyruvic transaminase (SGPT) and aspartate aminotransferase (AST)/SGOT ≤3 times the upper limit of normal (ULN), patients with liver metastasis ≤5 times ULN.
- An estimated creatinine clearance ≥40 mL/min using the Cockcroft Gault formula at Screening.
- Total bilirubin ≤2 mg/dL.
- Patients with Gilbert's Syndrome must have a total bilirubin ≤3 mg/dL.

**[002045]** 11. Patients were seronegative for the human immunodeficiency virus (HIV1 and HIV2). Patients with positive serology for hepatitis B virus surface antigen (HBsAg), hepatitis B core antibody (anti HBc), or hepatitis C virus (anti-HCV) indicating acute or chronic infection were enrolled depending on the viral load based on polymerase chain reaction (PCR) and the local prevalence of certain viral exposures.

**[002046]** 12. Patients had a washout period from prior anticancer therapy(ies) of a minimum duration, as detailed below prior to the first study treatment (*i.e.*, start of NMA-LD or pembrolizumab):

- Targeted therapy: prior targeted therapy with an epidermal growth factor receptor (EGFR), MEK, BRAF, ALK, ROS1 or other-targeted agents (*e.g.*, erlotinib, afatinib, dacomitinib, osimertinib, crizotinib, ceritinib, lorlatinib)

was allowed provided the washout is a minimum of 14 days prior to the start of treatment.

- Chemotherapy: adjuvant, neoadjuvant or definitive chemotherapy/ chemoradiation was allowed provided the washout is a minimum of 21 days prior to the start of treatment.
- Immunotherapy for Cohort 3B only, prior checkpoint-targeted therapy with an anti-PD-1, other mAbs, or vaccines were allowed with a washout period of ≥ 21 days before the start of NMA-LD.
- Palliative radiation therapy: prior external beam radiation was allowed provided all radiation-related toxicities were resolved to Grade 1 or baseline, excluding alopecia, skin pigmentation change, or other clinically insignificant events, *e.g.*, small area radiation dermatitis or rectal or urinary urgency
- The tumor lesion(s) being assessed as target for response via RECIST 1.1 were outside of the radiation portal; however, if within the portal, they must have demonstrated progression (see Inclusion Criterion above).
- Surgery/pre-planned procedure: previous surgical procedure(s) was permitted provided that wound healing had occurred, all complications had resolved, and at least 14 days have elapsed (for major operative procedures) prior to the tumor resection.

**[002047]** 13. Patients had recovered from all prior anticancer treatment-related adverse events (TRAEs) to Grade ≤1 (per Common Terminology Criteria for Adverse Events [CTCAE]), except for alopecia or vitiligo, prior to cohort assignment.

**[002048]** 14. Patients with stable Grade ≥2 toxicity from prior anticancer therapy were considered on a case by case basis after consultation with the Medical Monitor.

**[002049]** 15. Cohorts 1A, 2A, and 3A patients with irreversible toxicity not reasonably expected to be exacerbated by treatment with pembrolizumab were included only after consultation with the Medical Monitor. For patients in Cohort 3B only, patients with documented Grade ≥2 or higher diarrhea or colitis as a result of a previous treatment with immune checkpoint inhibitor CPI(s) must have been asymptomatic for at least 6 months or had a normal by visual assessment colonoscopy post-treatment prior to tumor resection.

**[002050]** 16. Patients must have provided written authorization for use and disclosure of protected health information.

**[002051]** *Exclusion Criteria*

**[002052]** Patients who meet ANY of the following criteria were excluded from the study:

**[002053]** 1. Patients with melanoma of uveal/ocular origin

**[002054]** 2. Patients who had received an organ allograft or prior cell transfer therapy that included a nonmyeloablative or myeloablative chemotherapy regimen within the past 20 years. (Note: This criterion was applicable for patients undergoing retreatment with TIL, with the exception that they had a prior NMA-LD regimen with their

**[002055]** prior TIL treatment.)

**[002056]** 3. Patients with symptomatic and/or untreated brain metastases.

- o • Patients with definitively-treated brain metastases will be considered for enrollment

**[002057]** after discussion with Medical Monitor; if, prior to the start of treatment the patient is

**[002058]** clinically stable for ≥2 weeks, there are no new brain lesions via magnetic resonance

**[002059]** imaging (MRI) post-treatment, and the patient does not require ongoing

**[002060]** corticosteroid treatment.

**[002061]** 4. Patients who are on a systemic steroid therapy within 21 days of enrollment.

**[002062]** 5. Patients who are pregnant or breastfeeding.

**[002063]** 6. Patients who had an active medical illness(es), which in the opinion of the Investigator, posed increased risks for study participation; such as systemic infections (*e.g.*, syphilis or any other infection requiring antibiotics), coagulation disorders, or other active major medical illnesses of the cardiovascular, respiratory, or immune systems.

**[002064]** 7. Patients may not have active or prior documented autoimmune or inflammatory disorders (including pneumonitis, inflammatory bowel disease [*e.g.*, colitis or Crohn's disease], diverticulitis [with the exception of diverticulosis], systemic lupus

erythematosus, sarcoidosis syndrome, or Wegener syndrome [granulomatosis with polyangiitis, Graves' disease, rheumatoid arthritis, hypophysitis, uveitis, etc.]). The following were exceptions to this criterion:

- Patients with vitiligo or alopecia.
- Patients with hypothyroidism (*e.g.*, following Hashimoto syndrome) stable on
- hormone replacement.
- Any chronic skin condition that did not require systemic therapy.
- Patients with celiac disease controlled by diet alone.

**[002065]** 8. Patients who had received a live or attenuated vaccination within 28 days prior to the start of treatment.

**[002066]** 9. Patients who had any form of primary immunodeficiency (such as severe combined immunodeficiency disease [SCID] and acquired immune deficiency syndrome [AIDS]).

**[002067]** 10. Patients with a history of hypersensitivity to any component of the study drugs. TILs were not administered to patients with a known hypersensitivity to any component of TIL product formulation including, but not limited to any of the following:

- • NMA-LD (cyclophosphamide, mesna, and fludarabine)
- • Proleukin®, aldesleukin, IL-2
- • Antibiotics of the aminoglycoside group (*i.e.*, streptomycin, gentamicin [excluding those who are skin-test negative for gentamicin hypersensitivity])
- • Any component of the TIL product formulation including dimethyl sulfoxide
- [DMSO], HSA, IL-2, and dextran-40
- • Pembrolizumab

**[002068]** 11. Patients who had a left ventricular ejection fraction (LVEF) <45% or who are New York Heart Association Class II or higher. A cardiac stress test demonstrating any irreversible wall movement abnormality in any patients ≥60 years of age or in patients who have a history of ischemic heart disease, chest pain, or clinically significant atrial and/or ventricular arrhythmias.

- Patients with an abnormal cardiac stress test could be enrolled if they had adequate ejection fraction and cardiology clearance with approval of the Sponsor's Medical Monitor.

**[002069]** 12. Patients who had obstructive or restrictive pulmonary disease and have a documented FEV1 (forced expiratory volume in 1 second) of ≤60% of predicted normal.

- • If a patient was not able to perform reliable spirometry due to abnormal upper airway anatomy (*i.e.*, tracheostomy), a 6-minute walk test was used to assess pulmonary function. Patients who were unable to walk a distance of at least 80% predicted for age and sex or demonstrates evidence of hypoxia at any point during the test (SpO2<90%) are excluded.

**[002070]** 13. Patients who had another primary malignancy within the previous 3 years (except for those which did not require treatment or had been curatively treated greater than 1 year ago, and in the judgment of the Investigator, did not pose a significant risk of recurrence including, but not limited to, non-melanoma skin cancer, DCIS, LCIS, prostate cancer Gleason score ≤6 or bladder cancer).

**[002071]** 14. Participation in another clinical study with an investigational product within 21 days of the initiation of treatment.

**[002072]** Study Endpoints and Planned Analyses

**[002073]** The primary and secondary endpoints were analyzed separately by cohort.

**[002074]** *Primary Endpoints:*

**[002075]** The ORR was defined as the proportion of patients who achieved either a confirmed PR or CR as best response as assessed by Investigators per RECIST 1.1 among the efficacy analysis set.

**[002076]** Objective response was evaluated per each disease assessment and the ORR was expressed as a binomial proportion with the corresponding 2-sided 90% CI. The primary analysis for each cohort occurred when all treated patients per cohort have an opportunity to be followed for 12 months, unless progressed/expired or discontinued early from the assessment period.

**[002077]** The safety primary endpoint was measured by any Grade 3 or higher TEAE incidence rate within each cohort expressed as binomial proportions with the corresponding 2-sided 90% CI.

**[002078]** *Secondary Endpoints:*

**[002079]** *Efficacy:*

**[002080]** The secondary efficacy endpoints were defined as follows:

**[002081]** CR rate as based on responders who achieved confirmed CR as assessed by Investigators. DCR was derived as the sum of the number of patients who achieved confirmed PR/CR or sustained SD (at least 6 weeks) divided by the number of patients in the efficacy analysis set × 100%. The CR rate and DCR was summarized using a point estimate and its 2-sided 90% CI.

**[002082]** DOR was defined among patients who achieved objective response. It was measured from the first-time response (PR/CR) criteria are met until the first date that recurrent or progressive disease was objectively documented, or receipt of subsequent anticancer therapy or the patient dies (whichever is first recorded). Patients not experiencing PD or have not died prior to the time of data cut or the final database lock will have their event times censored on the last date that an adequate assessment of tumor status is made.

**[002083]** PFS was defined as the time (in months) from the time of lymphodepletion to PD, or death due to any cause, whichever event is earlier. Patients not experiencing PD or not having expired at the time of the data cut or the final database lock had their event times censored on the last date that an adequate assessment of tumor status is made.

**[002084]** OS was defined as the time (in months) from the time of lymphodepletion to death due to any cause. Patients not having expired by the time of data cut or the final database lock had their event times censored on the last date of their known survival status.

**[002085]** DOR, PFS, and OS was subjected to right censoring. The Kaplan-Meier method will be used to summarize the time-to-event efficacy endpoints. The baseline data for the tumor assessment was the last scan before the lymphodepletion for all cohorts.

**[002086]** The above efficacy parameters will be estimated for applicable cohort for subsets defined by baseline disease characteristics; BRAF status (Cohort 1A only), HPV status (Cohort 2A only), squamous or non-squamous lung disease (Cohorts 3A and 3B only), and anti-PD-L1 status.

## EXAMPLE 18: A PHASE 2, MULTICENTER STUDY OF AUTOLOGOUS TUMOR INFILTRATING LYMPHOCYTES IN PATIENTS WITH SOLID TUMORS

**[002087]** The study in this example is a phase 2, multicenter, global open label study of autologous tumor infiltrating lymphocytes (TIL) in patients with select solid tumors (metastatic melanoma, head and neck squamous cell cancer, and non-small cell lung cancer (NSCLC)). The example uses the TIL product manufactured according to the Examples herein, including Examples 10-17, as well as dresibed in throughout the present application, which is cryopreserved and has a 22-day manufacturing process. A single infusion of TIL produce was given after patients had completed the preparatory regimen of non-myeloablative lymphodepletion with cyclophosphamide (60mg/kg x2 days) and fludarabine ($25mg^2/m$ x 5 days). There are 4 patient cohorts as described below:

- Cohort 1A (combination cohort): Stage IIIC or IV unresectable or metastatic melanoma patients who are immunotherapy naïve with ≤3 prior lines of systemic therapy. These patients will be receiving TIL product in combination with pembrolizumab.
- Cohort 2A (combination cohort): Advanced, recurrent or metastatic head and neck squamous cell carcinoma patients who are immunotherapy naïve with ≤3 prior lines of systemic therapy. These patients will be receiving TIL product in combination with pembrolizumab.
- Cohort 3A (combination cohort): Locally advanced or metastatic (Stage III-IV) NSCLC patients who are immunotherapy naïve with ≤3 prior lines of systemic therapy. These patients will be receiving TIL product in combination with pembrolizumab.
- Cohort 3B (single agent cohort): Stage III- IV NSCLC patients who have previously received systemic therapy with checkpoint inhibitors (anti-PD-1/anti-PD-L1) as part of ≤3 prior lines of systemic therapy. These patients will be receiving TIL product as single agent.

**[002088]** Two patients with NSCLC have been enrolled in Cohort 3B (TIL alone post immune checkpoint inhibitor) who had completed their first response assessment visit at day 42) are described below.

**[002089]** Patient A is a 63-year old female diagnosed with Stage IVA lung adenocarcinoma. She had received 3 prior lines of systemic therapy which included pembrolizumab, carboplatin/ bevacizumab and vinorelbine. Her best response to prior pembrolizumab therapy was progressive disease; to carboplatin/bevacizumab was a partial response, and her vinorelbine response was non-evaluable (she discontinued prior to response assessment). She underwent left lower lobe lung resection for TIL generation and was

infused with 3.75 x $10^9$ cells of TIL product. The patient had her first response assessment (Day 42) visit, at which computerized tomography (CT) scans showed a 4% reduction in the tumor load compared to baseline scans, which is a stable disease response per RECIST 1.1.

**[002090]** Patient B is a 70-year old female diagnosed with Stage IVB basaloid squamous cell carcinoma. She had received 3 prior lines of therapy which included carboplatin/abraxane, nivolumab and cisplatin/gemcitabine. Her best response to prior carbo/abraxane was not evaluable (therapy discontinued due to toxicity); to nivolumab was progressive disease and for cisplatin/gemcitabine was a partial response. She underwent splenic lesion resection for TIL generation and was infused with 39 x $10^9$ cells of TIL product. The patient had her first response assessment (Day 42) visit, at which CT scans showed a 44% reduction in the tumor load compared to baseline scans, which is a partial response per RECIST 1.1.

**[002091]** The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, systems and methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the art are intended to be within the scope of the following claims. All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains.

**[002092]** All headings and section designations are used for clarity and reference purposes only and are not to be considered limiting in any way. For example, those of skill in the art will appreciate the usefulness of combining various aspects from different headings and sections as appropriate according to the spirit and scope of the invention described herein.

**[002093]** All references cited herein are hereby incorporated by reference herein in their entireties and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

**[002094]** Many modifications and variations of this application can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments and examples described herein are offered by way of example only, and the application is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.

**WHAT IS CLAIMED IS:**

1. A method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor infiltrating lymphocytes (TILs) comprising the steps of:

   (a) obtaining and/or receiving a first population of TILs from surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells from a NSCLC tumor in a patient;

   (c) contacting the tumor fragments with a first cell culture medium;

   (d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

   (e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

   (f) harvesting the third population of TILs; and

   (g) administering a therapeutically effective portion of the third population of TILs to a patient with the NSCLC;

   wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

2. The method of Claim 1, wherein the obtaing the first population of TILs comprises a multilesional sampling method.

3. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 and/or anti-PD-L2 antibody.

4. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

5. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent.

6. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

7. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

8. The method of Claims 5 to 7, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

9. The method of Claim 1, wherein the refractory NSCLC has low expression of PD-L1.

10. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

11. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

12. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

13. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

14. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

15. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

16. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

17. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

18. The method of Claims 14 to 17, wherein bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane

or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

19. The method of Claim 1, wherein the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

20. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

21. The method of Claim 1, wherein the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

22. The method of Claim 1, wherein the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

23. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

24. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

25. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

26. The method of Claim 1, wherein the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

27. The method of Claim 1, wherein the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

28. The method of Claim 1, wherein the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

29. The method of any one of Claims 1 to 28, wherein the initial expansion is performed over a period of 21 days or less.

30. The method of any one of Claims 1 to 29, wherein the initial expansion is performed over

a period of 14 days or less.

31. The method of any one of Claims 1 to 30, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

32. The method of any one of Claims 1 to 31, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL in the second cell culture medium.

33. The method of any one of Claims 1 to 32, wherein the initial expansion is performed using a gas permeable container.

34. The method of any one of Claims 1 to 33, wherein the rapid expansion is performed using a gas permeable container.

35. The method of any one of Claims 1 to 34, wherein the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

36. The method of any one of Claims 1 to 35, wherein the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

37. The method of any one of Claims 1 to 36, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

38. The method of Claim 37, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

39. The method of any one of Claims 1 to 38, further comprising the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

40. The method of Claim 39, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

41. A method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor

infiltrating lymphocytes (TILs) comprising the steps of:

(a) resecting a tumor from a patient, the tumor comprising a first population of TILs;

(b) fragmenting the tumor into tumor fragments;

(c) contacting the tumor fragments with a first cell culture medium;

(d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

(e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

(f) harvesting the third population of TILs; and

(g) administering a therapeutically effective portion of the third population of TILs to a patient with the NSCLC;

wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

42. The method of Claim 41, wherein the tumor is resected from one or more tumor cites.

43. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 and/or anti-PD-L2 antibody.

44. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

45. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent.

46. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

47. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

48. The method of Claims 45 to 47, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

49. The method of Claim 41, wherein the refractory NSCLC has low expression of PD-L1.

50. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

51. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

52. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

53. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1.

54. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

55. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

56. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

57. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

58. The method of Claims 54 to 57, wherein said bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

59. The method of Claim 41, wherein the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

60. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

61. The method of Claim 41, wherein the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

62. The method of Claim 41, wherein the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

63. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

64. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

65. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

66. The method of Claim 41, wherein the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

67. The method of Claim 41, wherein the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

68. The method of Claim 41, wherein the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

69. The method of any one of Claims 41 to 68, wherein the initial expansion is performed over a period of 21 days or less.

70. The method of any one of Claims 41 to 69, wherein the initial expansion is performed over a period of 14 days or less.

71. The method of any one of Claims 41 to 70, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

72. The method of any one of Claims 41 to 71, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL in the second cell culture medium.

73. The method of any one of Claims 41 to 72, wherein the initial expansion is performed using a gas permeable container.

74. The method of any one of Claims 41 to 73, wherein the rapid expansion is performed using a gas permeable container.

75. The method of any one of Claims 41 to 74, wherein the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

76. The method of any one of Claims 41 to 75, wherein the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

77. The method of any one of Claims 41 to 76, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

78. The method of Claim 77, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

79. The method of any one of Claims 41 to 78, further comprising the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

80. The method of Claim 79, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

81. A method for treating a subject with non-small cell lung carcinoma (NSCLC), wherein the cancer is refractory to treatment with an anti-PD-1 antibody, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject into multiple

tumor fragments;

(b) adding the tumor fragments into a closed system;

(c) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;

(e) harvesting therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system; and

(f) transferring the harvested TIL population from step (e) to an infusion bag, wherein the transfer from step (e) to (f) occurs without opening the system;

(g) cryopreserving the infusion bag comprising the harvested TIL population from step (f) using a cryopreservation process; and

(h) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

82. The method of Claim 81, wherein the tumor sample is derived from a multilesional sampling method.

83. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

84. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

85. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent.

86. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated with a chemotherapeutic agent.

87. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

88. The method of Claims 85 to 87, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

89. The method of Claim 81, wherein the refractory NSCLC has low expression of PD-L1.

90. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

91. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

92. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

93. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

94. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

95. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

96. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

97. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

98. The method of Claims 94 to 97, wherein bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

99. The method of Claim 81, wherein the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

100. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

101. The method of Claim 81, wherein the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

102. The method of Claim 81, wherein the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

103. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

104. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

105. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

106. The method of Claim 81, wherein the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

107. The method of Claim 81, wherein the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

108. The method of Claim 81, wherein the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

109. The method of any one of Claims 81 to 108, wherein the initial expansion is

performed over a period of 21 days or less.

110. The method of any one of Claims 81 to 109, wherein the initial expansion is performed over a period of 14 days or less.

111. The method of any one of Claims 81 to 110, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

112. The method of any one of Claims 81 to 111, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL in the second cell culture medium.

113. The method of any one of Claims 81 to 112, wherein the initial expansion is performed using a gas permeable container.

114. The method of any one of Claims 81 to 113, wherein the rapid expansion is performed using a gas permeable container.

115. The method of any one of Claims 81 to 114, wherein the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

116. The method of any one of Claims 81 to 115, wherein the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

117. The method of any one of Claims 81 to 116, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

118. The method of Claim 117, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m$^2$/day for two days followed by administration of fludarabine at a dose of 25 mg/m$^2$/day for five days.

119. The method of any one of Claims 81 to 118, further comprising the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

120. The method of Claim 119, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof,

administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

121. The method according to any of the preceding Claims, wherein the NSCLC is refractory to a combination treatment or therapy comprising an anti-PD-1 and a chemotherapeutic agent.

122. The method of Claim 121, wherein the anti-PD-1 or the anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

123. The method according Claim 121, wherein the antiPD-1 is pembrolizumab.

124. The method according any one of Claims 121 to 123, wherein the chemotherapeutic agent is a platinum doublet chemotherapeutic agent(s).

125. The method according any one of Claims 121 to 123, wherein the platinum doublet therapy comprises:

i) a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin,

ii) and a second chemotherapeutic agent selected from the group consisting of vinorelbine, gemcitabine and a taxane (including for example, paclitaxel, docetaxel or nab-paclitaxel).

126. The method according any one of Claims 121 to 125, wherein the chemotherapeutic agent(s) is in combination with pemetrexed.

127. The method according any one of Claims 121 to 126, wherein the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, and cisplatin.

128. The method according any one of Claims 121 to 127, wherein the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, cisplatin, nivolumab, and ipilimumab.