CLOSED PROCESS FOR EXPANSION AND GENE EDITING OF TUMOR INFILTRATING LYMPHOCYTES AND USES OF SAME IN IMMUNOTHERAPY

FIELD OF THE INVENTION

[0001] Methods for expanding tumor infiltrating lymphocytes (TILs) and producing therapeutic populations of TILs are described herein. In addition, methods for gene-editing TILs, and uses of gene-edited TILs in the treatment of diseases such as cancer are disclosed herein.

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 processes are limited by length, cost, sterility concerns, and other factors described herein such that the potential to commercialize such processes is severely limited, and for these and other reasons, at the present time no commercial process has become available. There is an urgent need to provide TIL manufacturing processes and

therapies based on such processes that are appropriate for commercial scale manufacturing and regulatory approval for use in human patients at multiple clinical centers. Moreover, there is a strong need for more effective TIL therapies that can increase a patient's response rate and response robustness.

BRIEF SUMMARY OF THE INVENTION

[0004] The present invention provides methods for expanding TILs and producing therapeutic populations of TILs. According to exemplary embodiments, at least a portion of the therapeutic population of TILs are gene-edited to enhance their therapeutic effect.

[0005] In an embodiment, the present 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 patient by processing a tumor sample obtained from the patient 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, and optionally OKT-3, 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, optionally 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, 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 the therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system;

(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; and

(g) at any time during the method, gene-editing at least a portion of the TILs.

[0006] In some embodiments, the method further comprises the step of cryopreserving the infusion bag comprising the harvested TIL population in step (f) using a cryopreservation process.

[0007] In some embodiments, the cryopreservation process is performed using a 1:1 ratio of harvested TIL population to cryopreservation media.

[0008] In some embodiments, the antigen-presenting cells are peripheral blood mononuclear cells (PBMCs). In some embodiments, the PBMCs are irradiated and allogeneic. In some embodiments, the PBMCs are added to the cell culture on any of days 9 through 14 in step (d). In some embodiments, the antigen-presenting cells are artificial antigen-presenting cells.

[0009] In some embodiments, the harvesting in step (e) is performed using a membrane-based cell processing system.

[0010] In some embodiments, the harvesting in step (e) is performed using a LOVO cell processing system.

[0011] In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3.

[0012] 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.

[0013] In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3.

[0014] In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams.

[0015] In some embodiments, the cell culture medium is provided in a container selected from the group consisting of a G-container and a Xuri cellbag.

[0016] In some embodiments, the cell culture medium in step (d) further comprises IL-15 and/or IL-21.

[0017] In some embodiments, the IL-2 concentration is about 10,000 IU/mL to about 5,000 IU/mL.

[0018] In some embodiments, the IL-15 concentration is about 500 IU/mL to about 100 IU/mL.

[0019] In some embodiments, the IL-21 concentration is about 20 IU/mL to about 0.5 IU/mL.

[0020] In some embodiments, the infusion bag in step (f) is a HypoThermosol-containing infusion bag.

[0021] In some embodiments, the cryopreservation media comprises dimethlysulfoxide (DMSO). In some embodiments, the cryopreservation media comprises 7% to 10% dimethlysulfoxide (DMSO).

[0022] In some embodiments, the first period in step (c) and the second period in step (e) are each individually performed within a period of 10 days, 11 days, or 12 days.

[0023] In some embodiments, the first period in step (c) and the second period in step (e) are each individually performed within a period of 11 days.

[0024] In some embodiments, steps (a) through (f) are performed within a period of about 10 days to about 22 days.

[0025] In some embodiments, steps (a) through (f) are performed within a period of about 20 days to about 22 days.

[0026] In some embodiments, steps (a) through (f) are performed within a period of about 15 days to about 20 days.

[0027] In some embodiments, steps (a) through (f) are performed within a period of about 10 days to about 20 days.

[0028] In some embodiments, steps (a) through (f) are performed within a period of about 10 days to about 15 days.

[0029] In some embodiments, steps (a) through (f) are performed in 22 days or less.

[0030] In some embodiments, steps (a) through (f) are performed in 20 days or less.

[0031] In some embodiments, steps (a) through (f) are performed in 15 days or less.

[0032] In some embodiments, steps (a) through (f) are performed in 10 days or less.

[0033] In some embodiments, steps (a) through (f) and cryopreservation are performed in 22 days or less.

[0034] In some embodiments, the therapeutic population of TILs harvested in step (e) comprises sufficient TILs for a therapeutically effective dosage of the TILs.

[0035] In some embodiments, the number of TILs sufficient for a therapeutically effective dosage is from about 2.3×1010 to about 13.7×1010.

[0036] In some embodiments, steps (b) through (e) are performed in a single container, wherein performing steps (b) through (e) in a single container results in an increase in TIL yield per resected tumor as compared to performing steps (b) through (e) in more than one container.

[0037] In some embodiments, the antigen-presenting cells are added to the TILs during the second period in step (d) without opening the system.

[0038] In some embodiments, the third population of TILs in step (d) provides for increased efficacy, increased interferon-gamma production, increased polyclonality, increased average IP-10, and/or increased average MCP-1 when administered to a subject.

[0039] In some embodiments, the third population of TILs in step (d) provides for at least a five-fold or more interferon-gamma production when administered to a subject.

[0040] In some embodiments, the third population of TILs in step (d) 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 effector T cells and/or central memory T cells in the therapeutic population of TILs exhibit one or more characteristics selected from the group consisting of expressing CD27+, expressing CD28+, longer telomeres, increased CD57 expression, and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

[0041] In some embodiments, the effector T cells and/or central memory T cells obtained from the third population of TILs exhibit increased CD57 expression and decreased CD56 expression relative to effector T cells and/or central memory T cells obtained from the second population of cells.

[0042] In some embodiments, the risk of microbial contamination is reduced as compared to an open system.

[0043] In some embodiments, the TILs from step (g) are infused into a patient.

[0044] In some embodiments, the multiple fragments comprise about 4 fragments.

[0045] In some embodiments, the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

[0046] In some embodiments, the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[0047] In some embodiments, the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[0048] In some embodiments, the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

[0049] In some embodiments, the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

[0050] In some embodiments, the gene-editing is carried out after the first expansion and before the second expansion.

[0051] In some embodiments, the gene-editing is carried out before step (c), before step (d), or before step (e).

[0052] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

[0053] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

[0054] In some embodiments, the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

[0055] In some embodiments, the gene-editing 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.

[0056] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising 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.

[0057] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

[0058] In some embodiments, the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[0059] In some embodiments, the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

[0060] In some embodiments, the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

[0061] In some embodiments, the gene-editing comprises a CRISPR method.

[0062] In some embodiments, the CRISPR method is a CRISPR/Cas9 method.

[0063] In some embodiments, the gene-editing comprises a TALE method.

[0064] In some embodiments, the gene-editing comprises a zinc finger method.

[0065] In another embodiment, the present invention provides a method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the patient 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, and optionally OKT-3, 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, optionally 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, 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 the 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) optionally cryopreserving the infusion bag comprising the harvested TIL population from step (f) using a cryopreservation process;

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

(i) at any time during the method steps (a)-(f), gene-editing at least a portion of the TILs.

[0066] In some embodiments, the therapeutic population of TILs harvested in step (e) comprises sufficient TILs for administering a therapeutically effective dosage of the TILs in step (h).

[0067] In some embodiments, the number of TILs sufficient for administering a therapeutically effective dosage in step (h) is from about 2.3×1010 to about 13.7×1010.

[0068] In some embodiments, the antigen presenting cells (APCs) are PBMCs.

[0069] In some embodiments, the PBMCs are added to the cell culture on any of days 9 through 14 in step (d).

[0070] In some embodiments, prior to administering a therapeutically effective dosage of TIL cells in step (h), a non-myeloablative lymphodepletion regimen has been administered to the patient.

[0071] 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.

[0072] In some embodiments, the method further comprises the step of treating the patient with a high-dose IL-2 regimen starting on the day after administration of the TIL cells to the patient in step (h).

[0073] In some embodiments, 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.

[0074] In some embodiments, the third population of TILs in step (d) 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 effector T cells and/or central memory T cells in the therapeutic population of TILs exhibit one or more characteristics selected from the group consisting of expressing CD27+, expressing CD28+, longer telomeres, increased CD57 expression, and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

[0075] In some embodiments, the effector T cells and/or central memory T cells in the therapeutic population of TILs exhibit increased CD57 expression and decreased CD56 expression relative to effector T cells and/or central memory T cells obtained from the second population of cells.

[0076] 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)), renal cancer, and renal cell carcinoma.

[0077] In some embodiments, the cancer is selected from the group consisting of melanoma, HNSCC, cervical cancers, and NSCLC.

[0078] In some embodiments, the cancer is melanoma.

[0079] In some embodiments, the cancer is HNSCC.

[0080] In some embodiments, the cancer is a cervical cancer.

[0081] In some embodiments, the cancer is NSCLC.

[0082] In some embodiments, the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

[0083] In some embodiments, the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[0084] In some embodiments, the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[0085] In some embodiments, the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

[0086] In some embodiments, the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

[0087] In some embodiments, the gene-editing is carried out after the first expansion and before the second expansion.

[0088] In some embodiments, the gene-editing is carried out before step (c), before step (d), or before step (e).

[0089] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

[0090] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

[0091] In some embodiments, the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

[0092] In some embodiments, the gene-editing 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.

[0093] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising 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.

[0094] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

[0095] In some embodiments, the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[0096] In some embodiments, the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

[0097] In some embodiments, the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

[0098] In some embodiments, the gene-editing comprises a CRISPR method.

[0099] In some embodiments, the CRISPR method is a CRISPR/Cas9 method.

[00100] In some embodiments, the gene-editing comprises a TALE method.

[00101] In some embodiments, the gene-editing comprises a zinc finger method.

[00102] In another embodiment, the present invention provides a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

(a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;

(b) 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, 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 (a) to step (b) occurs without opening the system;

(c) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally 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, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;

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

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

(f) at any time during the method, gene-editing at least a portion of the TILs.

[00103] In some embodiments, the therapeutic population of TILs harvested in step (d) comprises sufficient TILs for a therapeutically effective dosage of the TILs.

[00104] In some embodiments, the number of TILs sufficient for a therapeutically effective dosage is from about 2.3×1010 to about 13.7×1010.

[00105] In some embodiments, the method further comprises the step of cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

[00106] In some embodiments, the cryopreservation process is performed using a 1:1 ratio of harvested TIL population to cryopreservation media.

[00107] In some embodiments, the antigen-presenting cells are peripheral blood mononuclear cells (PBMCs).

[00108] In some embodiments, the PBMCs are irradiated and allogeneic.

[00109] The method according to claim 68, wherein the PBMCs are added to the cell culture on any of days 9 through 14 in step (c).

[00110] In some embodiments, the antigen-presenting cells are artificial antigen-presenting cells.

[00111] In some embodiments, the harvesting in step (d) is performed using a LOVO cell processing system.

[00112] In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3.

[00113] 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.

[00114] In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3.

[00115] In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams.

[00116] In some embodiments, the multiple fragments comprise about 4 fragments.

[00117] In some embodiments, the second cell culture medium is provided in a container selected from the group consisting of a G-container and a Xuri cellbag.

[00118] In some embodiments, the infusion bag in step (e) is a HypoThermosol-containing infusion bag.

[00119] In some embodiments, the first period in step (b) and the second period in step (c) are each individually performed within a period of 10 days, 11 days, or 12 days.

[00120] In some embodiments, the first period in step (b) and the second period in step (c) are each individually performed within a period of 11 days.

[00121] In some embodiments, steps (a) through (e) are performed within a period of about 10 days to about 22 days.

[00122] In some embodiments, steps (a) through (e) are performed within a period of about 10 days to about 20 days.

[00123] In some embodiments, steps (a) through (e) are performed within a period of about 10 days to about 15 days.

[00124] In some embodiments, steps (a) through (e) are performed in 22 days or less.

[00125] In some embodiments, steps (a) through (e) and cryopreservation are performed in 22 days or less.

[00126] In some embodiments, steps (b) through (e) are performed in a single container, wherein performing steps (b) through (e) in a single container results in an increase in TIL yield per resected tumor as compared to performing steps (b) through (e) in more than one container.

[00127] In some embodiments, the antigen-presenting cells are added to the TILs during the second period in step (c) without opening the system.

[00128] In some embodiments, the third population of TILs in step (d) 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 effector T cells and/or central memory T cells obtained in the therapeutic population of TILs exhibit one or more characteristics selected from the group consisting of expressing CD27+, expressing CD28+, longer telomeres, increased CD57 expression, and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

[00129] In some embodiments, the effector T cells and/or central memory T cells obtained in the therapeutic population of TILs exhibit increased CD57 expression and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

[00130] In some embodiments, the risk of microbial contamination is reduced as compared to an open system.

[00131] In some embodiments, the TILs from step (e) are infused into a patient.

[00132] In some embodiments, the closed container comprises a single bioreactor.

[00133] In some embodiments, the closed container comprises a G-REX-10.

[00134] In some embodiments, the closed container comprises a G-REX -100.

[00135] In some embodiments, at step (d) the antigen presenting cells (APCs) are added to the cell culture of the second population of TILs at a APC:TIL ratio of 25:1 to 100:1.

[00136] In some embodiments, the cell culture has a ratio of 2.5×10^9 APCs to 100×10^6 TILs.

[00137] In some embodiments, at step (c) the antigen presenting cells (APCs) are added to the cell culture of the second population of TILs at a APC:TIL ratio of 25:1 to 100:1.

[00138] In some embodiments, the cell culture has ratio of 2.5×10^9 APCs to 100×10^6 TILs.

[00139] In some embodiments, the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

[00140] In some embodiments, the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[00141] In some embodiments, the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[00142] In some embodiments, the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

[00143] In some embodiments, the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

[00144] In some embodiments, the gene-editing is carried out after the first expansion and before the second expansion.

[00145] In some embodiments, the gene-editing is carried out before step (b), before step (c), or before step (d).

[00146] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

[00147] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

[00148] In some embodiments, the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

[00149] In some embodiments, the gene-editing 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.

[00150] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising 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.

[00151] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

[00152] In some embodiments, the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00153] In some embodiments, the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

[00154] In some embodiments, the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

[00155] In some embodiments, the gene-editing comprises a CRISPR method.

[00156] In some embodiments, the CRISPR method is a CRISPR/Cas9 method.

[00157] In some embodiments, the gene-editing comprises a TALE method.

[00158] In some embodiments, the gene-editing comprises a zinc finger method.

[00159] In another embodiment, the present invention provides a population of therapeutic TILs that have been expanded in accordance with any of the expansion methods described herein (e.g., for use in the treatment of a subject's cancer), wherein the population of therapeutic TILs has been permanently gene-edited.

[00160] In another embodiment, the present invention provides a population of expanded TILs for use in the treatment of a subject with cancer, wherein the population of expanded TILs is a third population of TILs obtainable by a method comprising:

(a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the patient 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, and optionally OKT-3, 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, optionally 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, 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 the therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system;

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

(h) at any time during the method, gene-editing at least a portion of the TILs.

[00161] In some embodiments, the above method further comprises one or more features recited in any of the methods and compositions described herein.

[00162] In some embodiments, the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

[00163] In some embodiments, the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[00164] In some embodiments, the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

[00165] In some embodiments, the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

[00166] In some embodiments, the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

[00167] In some embodiments, the gene-editing is carried out after the first expansion and before the second expansion.

[00168] In some embodiments, the gene-editing is carried out before step (c), before step (d), or before step (e).

[00169] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

[00170] In some embodiments, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

[00171] In some embodiments, the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

[00172] In some embodiments, the gene-editing 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.

[00173] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising 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.

[00174] In some embodiments, the one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

[00175] In some embodiments, the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00176] In some embodiments, the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

[00177] In some embodiments, the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

[00178] In some embodiments, the gene-editing comprises a CRISPR method.

[00179] In some embodiments, the CRISPR method is a CRISPR/Cas9 method.

[00180] In some embodiments, the gene-editing comprises a TALE method.

[00181] In some embodiments, the gene-editing comprises a zinc finger method.

[00182] In another embodiment, the present 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 patient by processing a tumor sample obtained from the patient 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 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 2 to 5 days;

(d) optionally adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

[00183] In another embodiment, the present invention provides a method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 2 to 5 days;

(d) optionally adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium; and

(k) administering a therapeutically effective dosage of the harvested TIL population from the infusion bag to the patient;

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like

Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

[00184] In another embodiment, the present invention provides a population of expanded TILs for use in the treatment of a subject with cancer, wherein the population of expanded TILs is a harvested population of TILs obtainable by a method comprising:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 2 to 5 days;

(d) optionally adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs

without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

[00185] In some embodiments, the method comprises performing the first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2, OKT-3 and a 4-1BB agonist antibody, wherein the OKT-3 and the 4-1BB agonist antibody are optionally present in the cell culture medium beginning on Day 0 or Day 1.

[00186] In another embodiment, the present invention provides a cryopreservation composition comprising the population of TILs for use to treat a subject with cancer, a cryoprotectant medium comprising DMSO, and an electrolyte solution.

[00187] In some embodiments, the cryopreservation composition may further comprise one or more stabilizers (e.g., HSA) and one or more lymphocyte growth factors (e.g., IL-2).

[00188] In some embodiments, the cryoprotectant medium comprising DMSO and the electrolyte solution are present in a ratio of about 1.1:1 to about 1:1.1.

[00189] In some embodiments, the cryopreservation composition comprises the cryoprotectant medium comprising DMSO in an amount of about 30 mL to about 70 mL, the electrolyte solution in an amount of about 30 mL to about 70 mL, HSA in an amount of about 0.1 g to about 1.0 g, and IL-2 in an amount of about 0.001 mg to about 0.005 mg.

BRIEF DESCRIPTION OF THE DRAWINGS

[00190] **Figure 1:** Shows a diagram of an embodiment of process 2A, a 22-day process for TIL manufacturing.

[00191] **Figure 2:** Shows a comparison between the 1C process and an embodiment of the 2A process for TIL manufacturing.

[00192] Figure 3: Shows the 1C process timeline.

[00193] Figure 4: Shows the process of an embodiment of TIL therapy using process 2A for TIL manufacturing, including administration and co-therapy steps, for higher cell counts.

[00194] Figure 5: Shows the process of an embodiment of TIL therapy using process 2A for TIL manufacturing, including administration and co-therapy steps, for lower cell counts.

[00195] Figure 6: Shows a detailed schematic for an embodiment of the 2A process.

[00196] Figures 7a, 7b and 7c: Depict the major steps of an embodiment of process 2A including the cryopreservation steps.

[00197] Figure 8: Depicts the clinical trial design including cohorts treated with process 1C and an embodiment of process 2A.

[00198] Figure 9: Exemplary Process 2A chart providing an overview of Steps A through F.

[00199] Figure 10: Process Flow Chart on Process 2A Data Collection Plan

[00200] Figure 11: Scheme of on exemplary embodiment of the Rapid Expansion Protocol (REP). Upon arrival the tumor is fragmented, placed into G-Rex flasks with IL-2 for TIL expansion (pre-REP expansion), for 11 days. For the triple cocktail studies, IL-2/IL-15/IL-21 is added at the initiation of the pre-REP. For the Rapid Expansion Protocol (REP), TIL are cultured with feeders and OKT3 for REP expansion for an additional 11 days.

[00201] Figure 12: Shows a diagram of an embodiment of process 2A, a 22-day process for TIL manufacturing.

[00202] Figure 13: Comparison table of Steps A through F from exemplary embodiments of process 1C and process 2A.

[00203] Figure 14: Detailed comparison of an embodiment of process 1C and an embodiment of process 2A.

[00204] Figure 15: Detailed scheme of an embodiment of a TIL therapy process.

[00205] Figure 16: Depiction of an embodiment of a cryopreserved TIL manufacturing process (22 days).

[00206] Figure 17: Table of process improvements from Gen 1 to Gen 2.

[00207] Figure 18: An embodiment of a TIL manufacturing process of the present invention.

[00208] Figure 19: Process Flow Chart of Process 2A.

[00209] Figure 20: Depiction of an embodiment of a TIL manufacturing process including electroporation step for use with gene-editing processes (including TALEN, zinc finger nuclease, and CRISPR methods as described herein).

[00210] Figure 21: Depiction of embodiments of TIL manufacturing processes including electroporation step for use with gene-editing processes (including TALEN, zinc finger nuclease, and CRISPR methods as described herein).

[00211] Figure 22: Depiction of 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.

[00212] Figure 23: Depiction of a TALEN construct that targets exon 2 of the Pdcd1 gene.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[00213] 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 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.

[00214] 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.

[00215] Previous papers report that limiting glycolysis and promoting mitochondrial metabolism in TILs prior to transfer is desirable as cells that are relying heavily on glycolysis will suffer nutrient deprivation upon adoptive transfer which results in a majority of the transferred cells dying. Thus, the art teaches that promoting mitochondrial metabolism might promote in vivo longevity and in fact suggests using inhibitors of glycolysis before induction of the immune response. See Chang, et al., *Nat. Immunol.* **2016,** 17(364).

[00216] The present invention is further directed in some embodiments to methods for evaluating and quantifying this increase in metabolic health. Thus, the present invention provides methods of assaying the relative health of a TIL population using one or more general evaluations of metabolism, including, but not limited to, rates and amounts of glycolysis, oxidative phosphorylation, spare respiratory capacity (SRC), and glycolytic reserve.

[00217] Furthermore, the present invention is further directed in some embodiments to methods for evaluating and quantifying this increase in metabolic health. Thus, the present invention provides methods of assaying the relative health of a TIL population using one or more general evaluations of metabolism, including, but not limited to, rates and amounts of glycolysis, oxidative phosphorylation, spare respiratory capacity (SRC), and glycolytic reserve.

[00218] In addition, optional additional evaluations include, but are not limited to, ATP production, mitochondrial mass and glucose uptake.

[00219] The present invention is further directed in some embodiments to enhancing the therapeutic effect of TILs with the use of gene editing technology. While adoptive transfer of tumor infiltrating lymphocytes (TILs) offers a promising and effective therapy, there is a strong need for more effective TIL therapies that can increase a patient's response rate and response robustness. As described herein, embodiments of the present invention provide methods for expanding TILs into a therapeutic population that is gene-edited to provide an enhanced therapeutic effect.

II. Definitions

[00220] 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.

[00221] The term "*in vivo*" refers to an event that takes place in a subject's body.

[00222] 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.

[00223] 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.

[00224] 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 outlined below.

[00225] 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.

[00226] By "population of cells" (including TILs) herein is meant a number of cells that share common traits. In general, populations generally range from 1×10^6 to 1×10^{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 1×10^8 cells. REP expansion is generally done to provide populations of 1.5×10^9 to 1.5×10^{10} cells for infusion.

[00227] 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.

[00228] 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.

[00229] 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.

[00230] 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.

[00231] 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.

[00232] 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.

[00233] 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.

[00234] 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.

[00235] 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. Preferably, the peripheral blood mononuclear cells are irradiated allogeneic peripheral blood mononuclear cells. PBMCs are a type of antigen-presenting cell.

[00236] 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.

[00237] 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. Anti-CD3 antibodies also include the UHCT1 clone (commercially available from BioLegend, San Diego, CA, USA), also known as T3 and CD3ε.

TABLE 1. Amino acid sequences of muromonab.

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

[00238] 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 recombinant human IL-2 (rhIL-2)	MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN RWITFCQSII STLT	60 120 134
SEQ ID NO:4 Aldesleukin	PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW ITFSQSIIST LT	60 120 132
SEQ ID NO:5 recombinant human IL-4 (rhIL-4)	MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI MREKYSKCSS	60 120 130
SEQ ID NO:6 recombinant human IL-7 (rhIL-7)	MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH	60 120 153
SEQ ID NO:7 recombinant human IL-15 (rhIL-15)	MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS	60 115
SEQ ID NO:8 recombinant human IL-21 (rhIL-21)	MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ HLSSRTHGSE DS	60 120 132

[00239] 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).

[00240] 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).

[00241] 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).

[00242] 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).

[00243] 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 (including 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.

[00244] The term "hematological malignancy" refers 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.

[00245] 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, but are not limited to, sarcomas, carcinomas, and lymphomas, such as cancers of the lung, breast, prostate, colon, rectum, and bladder. 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.

[00246] 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).

[00247] 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.

[00248] 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.

[00249] 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.

[00250] 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, at least one potassium channel agonist in combination with 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.

[00251] 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.

[00252] 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.

[00253] 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).

[00254] 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.

[00255] 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.

[00256] 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 9, including TILs referred to as reREP TILs).

[00257] 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.

[00258] 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 the 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.

[00259] 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.

[00260] 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."

III. Gene-Editing Processes

A. Overview: TIL Expansion + Gene-Editing

[00261] Embodiments of the present invention are directed to methods for expanding TIL populations, the methods comprising one or more steps of gene-editing at least a portion of the TILs in order to enhance their therapeutic effect. As used herein, "gene-editing," "gene editing," and "genome editing" refer to a type of genetic modification in which DNA is permanently modified in the genome of a cell, *e.g.*, DNA is inserted, deleted, modified or replaced within the cell's genome. In some embodiments, gene-editing causes the expression of a DNA sequence to be silenced (sometimes referred to as a gene knockout) or inhibited/reduced (sometimes referred to as a gene knockdown). In other embodiments, gene-editing causes the expression of a DNA sequence to be enhanced (*e.g.*, by causing over-expression). In accordance with embodiments of the present invention, gene-editing technology is used to enhance the effectiveness of a therapeutic population of TILs.

[00262] A method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., an exemplary TIL expansion method known as process 2A is described below), wherein the method further comprises gene-editing at least a portion of the TILs. According to additional embodiments, a method for expanding TILs into a therapeutic population of TILs is carried out in accordance with any embodiment of the methods described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, which are incorporated by reference herein in their entireties, wherein the method further comprises gene-editing at least a portion of the TILs. Thus, an embodiment of the present invention provides a therapeutic population of TILs that has been expanded in accordance with any embodiment described herein, wherein at least a portion of the therapeutic population has been gene-edited, *e.g.,* at least a portion of the therapeutic population of TILs that is transferred to the infusion bag is permanently gene-edited.

B. Timing of Gene-Editing During TIL Expansion

[00263] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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, and optionally OKT-3 (*e.g.*, OKT-3 may be present in the

culture medium beginning on the start date of the expansion process), 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, optionally 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, 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 the therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system;

(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; and

(g) at any time during the method, gene-editing at least a portion of the TILs.

[00264] As stated in step (g) of the embodiment described above, the gene-editing process may be carried out at any time during the TIL expansion method, which means that the gene editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(f) outlined in the method above, or before or after any of steps (a)-(f) outlined in the method above. According to certain embodiments, TILs are collected during the expansion method (e.g., the expansion method is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the expansion method (e.g., back into the culture medium) to continue the expansion process, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited. In an embodiment, the gene-editing process may be carried out before expansion by activating TILs, performing a gene-editing step on the activated TILs, and expanding the gene-edited TILs according to the processes described herein.

[00265] It should be noted that alternative embodiments of the expansion process may differ from the method shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(g), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two expansions, and it is possible that gene-editing may be conducted on the TILs during a third or fourth expansion, etc.

[00266] According to one embodiment, the gene-editing process is carried out on TILs from one or more of the first population, the second population, and the third population. For example, gene-editing may be carried out on the first population of TILs, or on a portion of TILs collected from the first population, and following the gene-editing process those TILs may subsequently be placed back into the expansion process (*e.g.,* back into the culture medium). Alternatively, gene-editing may be carried out on TILs from the second or third population, or on a portion of TILs collected from the second or third population, respectively, and following the gene-editing process those TILs may subsequently be placed back into the expansion process (*e.g.,* back into the culture medium). According to another embodiment, gene-editing is performed while the TILs are still in the culture medium and while the expansion is being carried out, *i.e.,* they are not necessarily "removed" from the expansion in order to conduct gene-editing.

[00267] According to another embodiment, the gene-editing process is carried out on TILs from the first expansion, or TILs from the second expansion, or both. For example, during the first expansion or second expansion, gene-editing may be carried out on TILs that are collected from the culture medium, and following the gene-editing process those TILs may subsequently be placed back into the expansion method, *e.g.*, by reintroducing them back into the culture medium.

[00268] According to another embodiment, the gene-editing process is carried out on at least a portion of the TILs after the first expansion and before the second expansion. For example, after the first expansion, gene-editing may be carried out on TILs that are collected from the culture medium, and following the gene-editing process those TILs may subsequently be placed back into the expansion method, *e.g.*, by reintroducing them back into the culture medium for the second expansion.

[00269] According to alternative embodiments, the gene-editing process is carried out before step (c) (*e.g.,* before, during, or after any of steps (a)-(b)), before step (d) (*e.g.,* before, during,

or after any of steps (a)-(c)), before step (e) (*e.g.,* before, during, or after any of steps (a)-(d)), or before step (f) (*e.g.,* before, during, or after any of steps (a)-(e)).

[00270] It should be noted with regard to OKT-3, according to certain embodiments, that the cell culture medium may comprise OKT-3 beginning on the start day (Day 0), or on Day 1 of the first expansion, such that the gene-editing is carried out on TILs after they have been exposed to OKT-3 in the cell culture medium on Day 0 and/or Day 1. According to another embodiment, the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium. Alternatively, the cell culture medium may comprise OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

[00271] It should also be noted with regard to a 4-1BB agonist, according to certain embodiments, that the cell culture medium may comprise a 4-1BB agonist beginning on the start day (Day 0), or on Day 1 of the first expansion, such that the gene-editing is carried out on TILs after they have been exposed to a 4-1BB agonist in the cell culture medium on Day 0 and/or Day 1. According to another embodiment, the cell culture medium comprises a 4-1BB agonist during the first expansion and/or during the second expansion, and the gene-editing is carried out before the 4-1BB agonist is introduced into the cell culture medium. Alternatively, the cell culture medium may comprise a 4-1BB agonist during the first expansion and/or during the second expansion, and the gene-editing is carried out after the 4-1BB agonist is introduced into the cell culture medium.

[00272] It should also be noted with regard to IL-2, according to certain embodiments, that the cell culture medium may comprise IL-2 beginning on the start day (Day 0), or on Day 1 of the first expansion, such that the gene-editing is carried out on TILs after they have been exposed to IL-2 in the cell culture medium on Day 0 and/or Day 1. According to another embodiment, the cell culture medium comprises IL-2 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the IL-2 is introduced into the cell culture medium. Alternatively, the cell culture medium may comprise IL-2 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the IL-2 is introduced into the cell culture medium.

[00273] As discussed above, one or more of OKT-3, 4-1BB agonist and IL-2 may be included in the cell culture medium beginning on Day 0 or Day 1 of the first expansion.

According to one embodiment, OKT-3 is included in the cell culture medium beginning on Day 0 or Day 1 of the first expansion, and/or a 4-1BB agonist is included in the cell culture medium beginning on Day 0 or Day 1 of the first expansion, and/or IL-2 is included in the cell culture medium beginning on Day 0 or Day 1 of the first expansion. According to an example, the cell culture medium comprises OKT-3 and a 4-1BB agonist beginning on Day 0 or Day 1 of the first expansion. According to another example, the cell culture medium comprises OKT-3, a 4-1BB agonist and IL-2 beginning on Day 0 or Day 1 of the first expansion. Of course, one or more of OKT-3, 4-1BB agonist and IL-2 may be added to the cell culture medium at one or more additional time points during the expansion process, as set forth in various embodiments described herein.

[00274] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third

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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium.

[00275] According to one embodiment, the foregoing method may be used to provide an autologous harvested TIL population for the treatment of a human subject with cancer.

C. Immune Checkpoints

[00276] According to particular embodiments of the present invention, a TIL population is gene-edited by genetically modifying one or more immune checkpoint genes in the TIL population. Stated another way, a DNA sequence within the TIL that encodes one or more of the TIL's immune checkpoints is permanently modified, *e.g.*, inserted, deleted or replaced, in the TIL's genome. Immune checkpoints are molecules expressed by lymphocytes that regulate an immune response via inhibitory or stimulatory pathways. In the case of cancer, immune checkpoint pathways are often activated to inhibit the anti-tumor response, *i.e.*, the expression of certain immune checkpoints by malignant cells inhibits the anti-tumor immunity and favors the growth of cancer cells. *See, e.g.,* Marin-Acevedo *et al., Journal of Hematology & Oncology* (2018) 11:39. Thus, certain inhibitory checkpoint molecules serve as targets for immunotherapies of the present invention. According to particular embodiments, TILs are gene-edited to block or stimulate certain immune checkpoint pathways and thereby enhance the body's immunological activity against tumors.

[00277] As used herein, an immune checkpoint gene comprises a DNA sequence encoding an immune checkpoint molecule. According to particular embodiments of the present invention, gene-editing TILs during the TIL expansion method 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. For example, gene-editing may cause the expression of an inhibitory receptor, such as PD-1 or CTLA-4, to be silenced or reduced in order to enhance an immune reaction.

[00278] The most broadly studied checkpoints include programmed cell death receptor-1 (PD-1) and cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), which are inhibitory receptors on immune cells that inhibit key effector functions (*e.g.*, activation, proliferation, cytokine release, cytoxicity, etc.) when they interact with an inhibitory ligand. Numerous checkpoint molecules, in addition to PD-1 and CTLA-4, have emerged as potential targets for immunotherapy, as discussed in more detail below.

[00279] Non-limiting examples of immune checkpoint genes that may be silenced or inhibited by permanently gene-editing TILs of the present invention include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, BAFF (BR3), 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. For example, immune checkpoint genes that may be silenced or inhibited in TILs of the present invention may be selected from the group comprising PD-1, CTLA-4, LAG-3, TIM-3, Cish, TGFβ, and PKA. BAFF (BR3) is described in Bloom, *et al.*, *J. Immunother.*, **2018,** in press. According to another example, immune checkpoint genes that may be silenced or inhibited in TILs of the present invention may be selected from the group comprising PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

[00280] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

1. PD-1

[00281] One of the most studied targets for the induction of checkpoint blockade is the programmed death receptor (PD1 or PD-1, also known as PDCD1), a member of the CD28 super family of T-cell regulators. Its ligands, PD-L1 and PD-L2, are expressed on a variety of tumor cells, including melanoma. The interaction of PD-1 with PD-L1 inhibits T-cell effector function, results in T-cell exhaustion in the setting of chronic stimulation, and induces T-cell apoptosis in the tumor microenvironment. PD1 may also play a role in tumor-specific escape from immune surveillance.

[00282] According to particular embodiments, expression of PD1 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of PD1. As described in more detail below, the gene-editing process may involve the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as PD1. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or reduce the expression of PD1 in the TILs.

2. CTLA-4

[00283] CTLA-4 expression is induced upon T-cell activation on activated T-cells, and competes for binding with the antigen presenting cell activating antigens CD80 and CD86. Interaction of CTLA-4 with CD80 or CD86 causes T-cell inhibition and serves to maintain balance of the immune response. However, inhibition of the CTLA-4 interaction with CD80 or CD86 may prolong T-cell activation and thus increase the level of immune response to a cancer antigen.

[00284] According to particular embodiments, expression of CTLA-4 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of CTLA-4. As described in more detail below, 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 an immune checkpoint gene, such as CTLA-4. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of CTLA-4 in the TILs.

3. LAG-3

[00285] Lymphocyte activation gene-3 (LAG-3, CD223) is expressed by T cells and natural killer (NK) cells after major histocompatibility complex (MHC) class II ligation. Although its mechanism remains unclear, its modulation causes a negative regulatory effect over T cell function, preventing tissue damage and autoimmunity. LAG-3 and PD-1 are frequently co-expressed and upregulated on TILs, leading to immune exhaustion and tumor growth. Thus, LAG-3 blockade improves anti-tumor responses. *See, e.g.,* Marin-Acevedo *et al., Journal of Hematology & Oncology* (2018) 11:39.

[00286] According to particular embodiments, expression of LAG-3 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of LAG-3. As described in more detail below, 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 an immune checkpoint gene, such as LAG-3. According to particular embodiments, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of LAG-3 in the TILs.

4. TIM-3

[00287] T cell immunoglobulin-3 (TIM-3) is a direct negative regulator of T cells and is expressed on NK cells and macrophages. TIM-3 indirectly promotes immunosuppression by inducing expansion of myeloid-derived suppressor cells (MDSCs). Its levels have been found to be particularly elevated on dysfunctional and exhausted T-cells, suggesting an important role in malignancy.

[00288] According to particular embodiments, expression of TIM-3 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For

example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of TIM-3. As described in more detail below, 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 an immune checkpoint gene, such as TIM-3. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of TIM-3 in the TILs.

5. Cish

[00289] Cish, a member of the suppressor of cytokine signaling (SOCS) family, is induced by TCR stimulation in CD8+ T cells and inhibits their functional avidity against tumors. Genetic deletion of Cish in CD8+ T cells may enhance their expansion, functional avidity, and cytokine polyfunctionality, resulting in pronounced and durable regression of established tumors. *See, e.g.,* Palmer *et al.*, *Journal of Experimental Medicine*, 212 (12): 2095 (2015).

[00290] According to particular embodiments, expression of Cish in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of Cish. As described in more detail below, 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 an immune checkpoint gene, such as Cish. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of Cish in the TILs.

6. TGFβ

[00291] The TGFβ signaling pathway has multiple functions in regulating cell growth, differentiation, apoptosis, motility and invasion, extracellular matrix production, angiogenesis, and immune response. TGFβ signaling deregulation is frequent in tumors and has crucial roles in tumor initiation, development and metastasis. At the microenvironment level, the TGFβ pathway contributes to generate a favorable microenvironment for tumor

growth and metastasis throughout carcinogenesis. *See, e.g.,* Neuzillet *et al.*, *Pharmacology & Therapeutics*, Vol. 147, pp. 22-31 (2015).

[00292] According to particular embodiments, expression of TGFβ in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or reducing the expression of TGFβ. As described in more detail below, 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 an immune checkpoint gene, such as TGFβ. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of TGFβ in the TILs.

[00293] In some embodiments, TGFβR2 (TGF beta receptor 2) may be suppressed by silencing TGFβR2 using a CRISPR/Cas9 system or by using a TGFβR2 dominant negative extracellular trap, using methods known in the art.

7. PKA

[00294] Protein Kinase A (PKA) is a well-known member of the serine-threonine protein kinase superfamily. PKA, also known as cAMP-dependent protein kinase, is a multi-unit protein kinase that mediates signal transduction of G-protein coupled receptors through its activation upon cAMP binding. It is involved in the control of a wide variety of cellular processes from metabolism to ion channel activation, cell growth and differentiation, gene expression and apoptosis. Importantly, PKA has been implicated in the initiation and progression of many tumors. *See, e.g.,* Sapio *et al.*, *EXCLI Journal*; 2014; 13: 843–855.

[00295] According to particular embodiments, expression of PKA in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of PKA. As described in more detail below, 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 an immune checkpoint gene, such as PKA. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of PKA in the TILs.

8. CBLB

[00296] CBLB (or CBL-B) is a E3 ubiquitin-protein ligase and is a negative regulator of T cell activation. Bachmaier, *et al., Nature*, 2000, 403, 211–216; Wallner, et al., *Clin. Dev. Immunol.* **2012,** 692639.

[00297] According to particular embodiments, expression of CBLB in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of CBLB. As described in more detail below, 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 an immune checkpoint gene, such as CBLB. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of PKA in the TILs. In some embodiments, CBLB is silenced using a TALEN knockout. In some embodiments, CBLB is silenced using a TALE-KRAB transcriptional inhibitor knock in. More details on these methods can be found in Boettcher and McManus, *Mol. Cell Review*, **2015,** *58,* 575-585.

9. TIGIT

[00298] T-cell immunoreceptor with Ig and ITIM (immunoreceptor tyrosine-based inhibitory motif) domain or TIGIT is a transmembrane glycoprotein receptor with an Ig-like V-type domain and an ITIM in its cytoplasmic domain. Khalil, *et al.*, *Advances in Cancer Research*, **2015**, 128, 1-68; Yu, *et al.*, *Nature Immunology*, **2009**, Vol. 10, No. 1, 48-57. TIGIT is expressed by some T cells and Natural Killer Cells. Additionally, TIGIT has been shown to be overexpressed on antigen-specific CD8+ T cells and CD8+ TILs, particularly from individuals with melanoma. Studies have shown that the TIGIT pathway contributes to tumor immune evasion and TIGIT inhibition has been shown to increase T-cell activation and proliferation in response to polyclonal and antigen-specific stimulation. Khalil, *et al.*, *Advances in Cancer Research*, **2015**, 128, 1-68. Further, coblockade of TIGIT with either

PD-1 or TIM3 has shown synergistic effects against solid tumors in mouse models. *Id.*; *see also* Kurtulus, *et al.*, *The Journal of Clinical Investigation*, **2015**, Vol. 125, No. 11, 4053-4062.

[00299] According to particular embodiments, expression of TIGIT in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of TIGIT. As described in more detail below, 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 an immune checkpoint gene, such as TIGIT. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of TIGIT in the TILs.

D. Overexpression of Co-Stimulatory Receptors or Adhesion Molecules

[00300] According to additional embodiments, gene-editing TILs during the TIL expansion method causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs. For example, gene-editing may cause the expression of a stimulatory receptor to be enhanced, which means that it is overexpressed as compared to the expression of a stimulatory receptor that has not been genetically modified. Non-limiting examples of immune checkpoint genes that may exhibit enhanced expression by permanently gene-editing TILs of the present invention include certain chemokine receptors and interleukins, such as CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

1. CCRs

[00301] For adoptive T cell immunotherapy to be effective, T cells need to be trafficked properly into tumors by chemokines. A match between chemokines secreted by tumor cells, chemokines present in the periphery, and chemokine receptors expressed by T cells is important for successful trafficking of T cells into a tumor bed.

[00302] According to particular embodiments, gene-editing methods of the present invention may be used to increase the expression of certain chemokine receptors in the TILs, such as one or more of CCR2, CCR4, CCR5, CXCR2, CXCR3 and CX3CR1. Over-expression of CCRs may help promote effector function and proliferation of TILs following adoptive transfer.

[00303] According to particular embodiments, expression of one or more of CCR2, CCR4, CCR5, CXCR2, CXCR3 and CX3CR1 in TILs is enhanced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by enhancing the expression of one or more of CCR2, CCR4, CCR5, CXCR2, CXCR3 and CX3CR1. As described in more detail below, 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 a chemokine receptor gene. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to enhance the expression of certain chemokine receptors in the TILs.

[00304] In an embodiment, CCR4 and/or CCR5 adhesion molecules are inserted into a TIL population using a gamma-retroviral or lentiviral method as described herein. In an embodiment, CXCR2 adhesion molecule are inserted into a TIL population using a gamma-retroviral or lentiviral method as described in Forget, *et al., Frontiers Immunology* **2017**, *8*, 908 or Peng, *et al.*, *Clin. Cancer Res.* **2010**, *16*, 5458, the disclosures of which are incorporated by reference herein.

[00305] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1 and, optionally, LAG-3, and further wherein a CXCR2 adhesion molecule is inserted by a gammaretroviral or lentiviral method into the first population of TILs, second population of TILs, or harvested population of TILs.

[00306] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1 and, optionally, LAG-3, and further wherein a CCR4 and/or CCR5 adhesion molecule is inserted by a gammaretroviral or lentiviral method into the first population of TILs, second population of TILs, or harvested population of TILs.

[00307] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1 and, optionally, LAG-3, and further wherein 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.

2. Interleukins

[00308] According to additional embodiments, gene-editing methods of the present invention may be used to increase the expression of certain interleukins, such as one or more of IL-2, IL-4, IL-7, IL-10, IL-15, and IL-21. Certain interleukins have been demonstrated to augment effector functions of T cells and mediate tumor control.

[00309] According to particular embodiments, expression of one or more of IL-2, IL-4, IL-7, IL-10, IL-15, and IL-21 in TILs is enhanced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by enhancing the expression of one or more of IL-2, IL-4, IL-7, IL-10, IL-15, and IL-21. As described in more detail below, 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 an interleukin gene. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to enhance the expression of certain interleukins in the TILs.

[00310] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1 and, optionally, LAG-3, and further wherein a interleukin selected from the group consisting of IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, 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. Gene Editing Methods

[00311] 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.

[00312] In some embodiments, 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 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.

[00313] 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 or 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.

[00314] 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.

[00315] 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.

[00316] 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, embodiments of 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., 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 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.

[00317] 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.

1. CRISPR Methods

[00318] 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 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.

[00319] 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. CRISPR systems can be divided into two main classes, Class 1 and Class 2, which are further classified into different types and sub-types. The classification of the CRISPR systems is based on the effector Cas proteins that are capable of cleaving specific nucleic acids. In Class 1 CRISPR systems the effector module consists of a multi-protein complex, whereas Class 2 systems only use one effector protein. Class 1 CRISPR includes Types I, III, and IV and Class 2 CRISPR includes Types II, V, and VI. While any of these types of CRISPR systems may be used in accordance with the present invention, there are three types of CRISPR systems which incorporate RNAs and Cas proteins that are preferred for use in accordance with the present invention: Types I (exemplified by Cas3), II (exemplified by Cas9), and III (exemplified by Cas10). The Type II CRISPR is one of the most well-characterized systems.

[00320] 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. Thus, according to certain embodiments, Cas9 serves as

an RNA-guided DNA endonuclease that cleaves DNA upon crRNA-tracrRNA recognition. 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 sgRNA is a synthetic RNA that includes a scaffold sequence necessary for Cas-binding and a user-defined approximately 17- to 20-nucleotide spacer that defines the genomic target to be modified. Thus, a user can change the genomic target of the Cas protein by changing the target sequence present in the sgRNA. The CRISPR/Cas system is directly portable to human cells by co-delivery of plasmids expressing the Cas9 endo-nuclease and the RNA components (e.g., sgRNA). Different variants of Cas proteins may be used to reduce targeting limitations (e.g., orthologs of Cas9, such as Cpf1).

[00321] According to one embodiment, an engineered, programmable, non-naturally occurring Type II CRISPR-Cas system comprises a Cas9 protein and at least one guide RNA that targets and hybridizes to a target sequence of a DNA molecule in a TIL, wherein the DNA molecule encodes and the TIL expresses at least one immune checkpoint molecule and the Cas9 protein cleaves the DNA molecules, whereby expression of the at least one immune checkpoint molecule is altered; and, wherein the Cas9 protein and the guide RNA do not naturally occur together. According to an embodiment, the expression of two or more immune checkpoint molecules is altered. According to an embodiment, the guide RNA(s) comprise a guide sequence fused to a tracr sequence. For example, the guide RNA may comprise crRNA-tracrRNA or sgRNA. According to aspects of the present invention, the terms "guide RNA", "single guide RNA" and "synthetic guide RNA" may be used interchangeably and refer to the polynucleotide sequence comprising the guide sequence, which is the approximately 17-20 bp sequence within the guide RNA that specifies the target site.

[00322] Variants of Cas9 having improved on-target specificity compared to Cas9 may also be used in accordance with embodiments of the present invention. Such variants may be referred to as high-fidelity Cas-9s. According to an embodiment, a dual nickase approach may be utilized, wherein two nickases targeting opposite DNA strands generate a DSB within the target DNA (often referred to as a double nick or dual nickase CRISPR system). For example, this approach may involve the mutation of one of the two Cas9 nuclease domains, turning Cas9 from a nuclease into a nickase. Non-limiting examples of high-fidelity Cas9s include eSpCas9, SpCas9-HF1 and HypaCas9. Such variants may reduce or eliminate unwanted changes at non-target DNA sites. *See, e.g.*, Slaymaker IM, et al. *Science*. **2015** Dec

1, Kleinstiver BP, et al. *Nature*. **2016** Jan 6, and Ran et al., *Nat Protoc.* 2013 Nov; 8(11):2281-2308, the disclosures of which are incorporated by reference herein.

[00323] Additionally, according to particular embodiments, Cas9 scaffolds may be used that improve gene delivery of Cas9 into cells and improve on-target specificity, such as those disclosed in U.S. Patent Application Publication No. 2016/0102324, which is incorporated by reference herein. For example, Cas9 scaffolds may include a RuvC motif as defined by (D-[I/L]-G-X-X-S-X-G-W-A) and/or a HNH motif defined by (Y-X-X-D-H-X-X-P-X-S-X-X-X-D-X-S), where X represents any one of the 20 naturally occurring amino acids and [I/L] represents isoleucine or leucine. The HNH domain is responsible for nicking one strand of the target dsDNA and the RuvC domain is involved in cleavage of the other strand of the dsDNA. Thus, each of these domains nick a strand of the target DNA within the protospacer in the immediate vicinity of PAM, resulting in blunt cleavage of the DNA. These motifs may be combined with each other to create more compact and/or more specific Cas9 scaffolds. Further, the motifs may be used to create a split Cas9 protein (i.e., a reduced or truncated form of a Cas9 protein or Cas9 variant that comprises either a RuvC domain or a HNH domain) that is divided into two separate RuvC and HNH domains, which can process the target DNA together or separately.

[00324] According to particular embodiments, a CRISPR method comprises silencing or reducing the expression of one or more immune checkpoint genes in TILs by introducing a Cas9 nuclease and a guide RNA (e.g., crRNA-tracrRNA or sgRNA) containing a sequence of approximately 17-20 nucleotides specific to a target DNA sequence of the immune checkpoint gene(s). The guide RNA may be delivered as RNA or by transforming a plasmid with the guide RNA-coding sequence under a promoter. The CRISPR/Cas enzymes introduce a double-strand break (DSB) at a specific location based on a sgRNA-defined target sequence. DSBs may be repaired in the cells by non-homologous end joining (NHEJ), a mechanism which frequently causes insertions or deletions (indels) in the DNA. Indels often lead to frameshifts, creating loss of function alleles; for example, by causing premature stop codons within the open reading frame (ORF) of the targeted gene. According to certain embodiments, the result is a loss-of-function mutation within the targeted immune checkpoint gene.

[00325] Alternatively, DSBs induced by CRISPR/Cas enzymes may be repaired by homology-directed repair (HDR) instead of NHEJ. While NHEJ-mediated DSB repair often disrupts the open reading frame of the gene, homology directed repair (HDR) can be used to

generate specific nucleotide changes ranging from a single nucleotide change to large insertions. According to an embodiment, HDR is used for gene editing immune checkpoint genes by delivering a DNA repair template containing the desired sequence into the TILs with the sgRNA(s) and Cas9 or Cas9 nickase. The repair template preferably contains the desired edit as well as additional homologous sequence immediately upstream and downstream of the target gene (often referred to as left and right homology arms).

[00326] According to particular embodiments, an enzymatically inactive version of Cas9 (deadCas9 or dCas9) may be targeted to transcription start sites in order to repress transcription by blocking initiation. Thus, targeted immune checkpoint genes may be repressed without the use of a DSB. A dCas9 molecule retains the ability to bind to target DNA based on the sgRNA targeting sequence. According to an embodiment of the present invention, a CRISPR method comprises silencing or reducing the expression of one or more immune checkpoint genes by inhibiting or preventing transcription of the targeted gene(s). For example, a CRISPR method may comprise fusing a transcriptional repressor domain, such as a Kruppel-associated box (KRAB) domain, to an enzymatically inactive version of Cas9, thereby forming, *e.g.*, a dCas9-KRAB, that targets the immune checkpoint gene's transcription start site, leading to the inhibition or prevention of transcription of the gene. Preferably, the repressor domain is targeted to a window downstream from the transcription start site, *e.g.*, about 500 bp downstream. This approach, which may be referred to as CRISPR interference (CRISPRi), leads to robust gene knockdown via transcriptional reduction of the target RNA.

[00327] According to particular embodiments, an enzymatically inactive version of Cas9 (deadCas9 or dCas9) may be targeted to transcription start sites in order to activate transcription. This approach may be referred to as CRISPR activation (CRISPRa). According to an embodiment, a CRISPR method comprises increasing the expression of one or more immune checkpoint genes by activating transcription of the targeted gene(s). According to such embodiments, targeted immune checkpoint genes may be activated without the use of a DSB. A CRISPR method may comprise targeting transcriptional activation domains to the transcription start site; for example, by fusing a transcriptional activator, such as VP64, to dCas9, thereby forming, *e.g.*, a dCas9-VP64, that targets the immune checkpoint gene's transcription start site, leading to activation of transcription of the gene. Preferably, the activator domain is targeted to a window upstream from the transcription start site, *e.g.*, about 50-400 bp downstream

[00328] Additional embodiments of the present invention may utilize activation strategies that have been developed for potent activation of target genes in mammalian cells. Non-limiting examples include co-expression of epitope-tagged dCas9 and antibody-activator effector proteins (*e.g.*, the SunTag system), dCas9 fused to a plurality of different activation domains in series (*e.g.,* dCas9-VPR) or co-expression of dCas9-VP64 with a modified scaffold gRNA and additional RNA-binding helper activators (*e.g.,* SAM activators).

[00329] According to other embodiments, a CRISPR-mediated genome editing method referred to as CRISPR assisted rational protein engineering (CARPE) may be used in accordance with embodiments of the present invention, as disclosed in US Patent No. 9,982,278, which is incorporated by reference herein. CARPE involves the generation of "donor" and "destination" libraries that incorporate directed mutations from single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) editing cassettes directly into the genome. Construction of the donor library involves cotransforming rationally designed editing oligonucleotides into cells with a guide RNA (gRNA) that hybridizes to a target DNA sequence. The editing oligonucleotides are designed to couple deletion or mutation of a PAM with the mutation of one or more desired codons in the adjacent gene. This enables the entire donor library to be generated in a single transformation. The donor library is retrieved by amplification of the recombinant chromosomes, such as by a PCR reaction, using a synthetic feature from the editing oligonucleotide, namely, a second PAM deletion or mutation that is simultaneously incorporated at the 3' terminus of the gene. This covalently couples the codon target mutations directed to a PAM deletion. The donor libraries are then co-transformed into cells with a destination gRNA vector to create a population of cells that express a rationally designed protein library.

[00330] According to other embodiments, methods for trackable, precision genome editing using a CRISPR-mediated system referred to as Genome Engineering by Trackable CRISPR Enriched Recombineering (GEn-TraCER) may be used in accordance with embodiments of the present invention, as disclosed in US Patent No. 9,982,278, which is incorporated by reference herein. The GEn-TraCER methods and vectors combine an editing cassette with a gene encoding gRNA on a single vector. The cassette contains a desired mutation and a PAM mutation. The vector, which may also encode Cas9, is the introduced into a cell or population of cells. This activates expression of the CRISPR system in the cell or population of cells, causing the gRNA to recruit Cas9 to the target region, where a dsDNA break occurs, allowing integration of the PAM mutation.

[00331] 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.

[00332] 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, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00333] 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.

[00334] 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 9,790,490, the disclosure of which is incorporated by reference herein. The CRISPR/Cpf1 system is functionally distinct from the CRISPR-Cas9 system in that Cpf1-associated CRISPR arrays are processed into mature crRNAs without the need for an additional tracrRNA. The crRNAs used in the CRISPR/Cpf1 system have a spacer or guide sequence and a direct repeat sequence. The Cpf1p-crRNA complex that is formed using this method is sufficient by itself to cleave the target DNA.

[00335] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR)/Cas9 or CRISPR/Cpf1 system for modulating the expression of at least one protein.

[00336] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR)/Cas9 or CRISPR/Cpf1 system for inhibiting the expression of PD-1 and LAG-3.

2. TALE Methods

[00337] 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.

[00338] 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.

[00339] Several large, systematic studies utilizing various assembly methods have indicated that TALE repeats can be combined to recognize virtually any user-defined sequence. Strategies that enable the rapid assembly of custom TALE arrays include Golden Gate

molecular cloning, high-throughput solid-phase assembly, and ligation-independent cloning techniques. 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). Additionally web-based tools, such as TAL Effector-Nucleotide Target 2.0, are available that enable the design of custom TAL effector repeat arrays for desired targets and also provides predicted TAL effector binding sites. *See* Doyle, *et al.*, *Nucleic Acids Research*, **2012**, Vol. 40, W117-W122. Examples of 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.

[00340] According to an embodiment of the present invention, a TALE method comprises silencing or reducing the expression of one or more immune checkpoint genes by inhibiting or preventing transcription of the targeted gene(s). For example, a TALE method may include utilizing KRAB-TALEs, wherein the method comprises fusing a transcriptional Kruppel-associated box (KRAB) domain to a DNA binding domain that targets the gene's transcription start site, leading to the inhibition or prevention of transcription of the gene.

[00341] According to another embodiment, a TALE method comprises silencing or reducing the expression of one or more immune checkpoint genes by introducing mutations in the targeted gene(s). For example, a TALE method may include fusing a nuclease effector domain, such as FokI, to the TALE DNA binding domain, resulting in a TALEN. FokI is active as a dimer; hence, the method comprises constructing pairs of TALENs to position the FOKL nuclease domains to adjacent genomic target sites, where they introduce DNA double strand breaks. A double strand break may be completed following correct positioning and dimerization of FokI. Once the double strand break is introduced, DNA repair can be achieved via two different mechanisms: the high-fidelity homologous recombination pair (HRR) (also known as homology-directed repair or HDR) or the error-prone non-homologous end joining (NHEJ). Repair of double strand breaks via NHEJ preferably results in DNA target site deletions, insertions or substitutions, *i.e.*, NHEJ typically leads to the introduction of small insertions and deletions at the site of the break, often inducing frameshifts that knockout gene function. According to particular embodiments, the TALEN pairs are targeted to the most 5' exons of the genes, promoting early frame shift mutations or premature stop codons. The genetic mutation(s) introduced by TALEN are preferably permanent. Thus,

according to one embodiment, the method comprises silencing or reducing expression of an immune checkpoint gene by utilizing dimerized TALENs to induce a site-specific double strand break that is repaired via error-prone NHEJ, leading to one or more mutations in the targeted immune checkpoint gene.

[00342] According to additional embodiments, TALENs are utilized to introduce genetic alterations via HRR, such as non-random point mutations, targeted deletion, or addition of DNA fragments. The introduction of DNA double strand breaks enables gene editing via homologous recombination in the presence of suitable donor DNA. According to an embodiment, the method comprises co-delivering dimerized TALENs and a donor plasmid bearing locus-specific homology arms to induce a site-specific double strand break and integrate one or more transgenes into the DNA.

[00343] According to another embodiment, a TALEN that is a hybrid protein derived from FokI and AvrXa7, as disclosed in U.S. Patent Publication No. 2011/0201118, may be used in accordance with embodiments of the present invention. This TALEN retains recognition specificity for target nucleotides of AvrXa7 and the double-stranded DNA cleaving activity of FokI. The same methods can be used to prepare other TALEN having different recognition specificity. For example, compact TALENs may be generated by engineering a core TALE scaffold having different sets of RVDs to change the DNA binding specificity and target a specific single dsDNA target sequence. *See* U.S. Patent Publication No. 2013/0117869. A selection of catalytic domains can be attached to the scaffold to effect DNA processing, which may be engineered to ensure that the catalytic domain is capable of processing DNA near the single dsDNA target sequence when fused to the core TALE scaffold. A peptide linker may also be engineered to fuse the catalytic domain to the scaffold to create a compact TALEN made of a single polypeptide chain that does not require dimerization to target a specific single dsDNA sequence. A core TALE scaffold may also be modified by fusing a catalytic domain, which may be a TAL monomer, to its N-terminus, allowing for the possibility that this catalytic domain might interact with another catalytic domain fused to another TAL monomer, thereby creating a catalytic entity likely to process DNA in the proximity of the target sequences. *See* U.S. Patent Publication No. 2015/0203871. This architecture allows only one DNA strand to be targeted, which is not an option for classical TALEN architectures.

[00344] According to an embodiment of the present invention, conventional RVDs may be used create TALENs that are capable of significantly reducing gene expression. In an

embodiment, four RVDs, NI, HD, NN, and NG, are used to target adenine, cytosine, guanine, and thymine, respectively. These conventional RVDs can be used to, for instance, create TALENs targeting the the PD-1 gene. Examples of TALENs using conventional RVDs include the T3v1 and T1 TALENs disclosed in Gautron *et al.*, *Molecular Therapy: Nucleic Acids* Dec. 2017, Vol. 9:312-321 (Gautron), which is incorporated by reference herein. The T3v1 and T1 TALENs target the second exon of the *PDCD1* locus where the PD-L1 binding site is located and are able to considerably reduce PD-1 production. In an embodiment, the T1 TALEN does so by using target SEQ ID NO:127 and the T3v1 TALEN does so by using target SEQ ID NO:128.

[00345] According to another embodiment, TALENs are modified using non-conventional RVDs to improve their activity and specificity for a target gene, such as disclosed in Gautron. Naturally occurring RVDs only cover a small fraction of the potential diversity repertoire for the hypervariable amino acid locations. Non-conventional RVDs provide an alternative to natural RVDs and have novel intrinsic targeting specificity features that can be used to exclude the targeting of off-site targets (sequences within the genome that contain a few mismatches relative to the targeted sequence) by TALEN. Non-conventional RVDs may be identified by generating and screening collections of TALEN containing alternative combinations of amino acids at the two hypervariable amino acid locations at defined positions of an array as disclosed in Juillerat, *et al.*, *Scientific Reports* 5, Article Number 8150 (2015), which is incorporated by reference herein. Next, non-conventional RVDs may be selected that discriminate between the nucleotides present at the position of mismatches, which can prevent TALEN activity at off-site sequences while still allowing appropriate processing of the target location. The selected non-conventional RVDs may then be used to replace the conventional RVDs in a TALEN. Examples of TALENs where conventional RVDs have been replaced by non-conventional RVDs include the T3v2 and T3v3 PD-1 TALENs produced by Gautron. These TALENs had increased specificity when compared to TALENs using conventional RVDs.

[00346] According to additional embodiments, TALEN may be utilized to introduce genetic alterations to silence or reduce the expression of two genes. For instance, two separate TALEN may be generated to target two different genes and then used together. The molecular events generated by the two TALEN at their respective loci and potential off-target sites may be characterized by high-throughput DNA sequencing. This enables the analysis of off-target sites and identification of the sites that might result from the use of both TALEN.

Based on this information, appropriate conventional and non-conventional RVDs may be selected to engineer TALEN that have increased specificity and activity even when used together. For example, Gautron discloses the combined use of T3v4 PD-1 and TRAC TALEN to produce double knockout CAR T cells, which maintained a potent *in vitro* anti-tumor function.

[00347] In an embodiment, the method of Gautron or other methods described herein may be employed to genetically-edit TILs, which may then be expanded by any of the procedures described herein. In an embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises the steps of:

(a) activating a first population of TILs obtained from a tumor resected from a patient using CD3 and CD28 activating beads or antibodies for 1 to 5 days;

(b) gene-editing at least a portion of the first population of TILs using electroporation of transcription activator-like effector nucleases to obtain a second population of TILs;

(c) optionally incubating the second population of TILs;

(d) performing a first expansion by culturing the second population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a third 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 to 14 days to obtain the third population of TILs;

(e) performing a second expansion by supplementing the cell culture medium of the third population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 7 to 14 days to obtain the fourth population of TILs, wherein the fourth population of TILs is a therapeutic population of TILs;

(f) harvesting the therapeutic population of TILs obtained from step (e);

(g) 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; and

(h) wherein one or more of steps (a) to (g) are performed in a closed, sterile system.

[00348] In an embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises the steps of:

(a) activating a first population of TILs obtained from a tumor resected from a patient using CD3 and CD28 activating beads or antibodies for 1 to 5 days;

(b) gene-editing at least a portion of the first population of TILs using electroporation of transcription activator-like effector nucleases in cytoporation medium to obtain a second population of TILs;

(c) optionally incubating the second population of TILs;

(d) performing a first expansion by culturing the second population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a third 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 6 to 9 days to obtain the third population of TILs;

(e) performing a second expansion by supplementing the cell culture medium of the third population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 9 to 11 days to obtain the fourth population of TILs, wherein the fourth population of TILs is a therapeutic population of TILs;

(f) harvesting the therapeutic population of TILs obtained from step (e);

(g) 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; and

(h) wherein one or more of steps (a) to (g) are performed in a closed, sterile system.

[00349] In an embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises the steps of:

(a) activating a first population of TILs obtained from a tumor resected from a patient using CD3 and CD28 activating beads or antibodies for 1 to 5 days;

(b) gene-editing at least a portion of the first population of TILs using electroporation of transcription activator-like effector nucleases in cytoporation medium to obtain a second population of TILs;

(c) optionally incubating the second population of TILs, wherein the incubation is performed at about 30-40 °C with about 5% CO_2;

(d) performing a first expansion by culturing the second population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a third 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 6 to 9 days to obtain the third population of TILs;

(e) performing a second expansion by supplementing the cell culture medium of the

third population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 9 to 11 days to obtain the fourth population of TILs, wherein the fourth population of TILs is a therapeutic population of TILs;

(f) harvesting the therapeutic population of TILs obtained from step (e);

(g) 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; and

(h) wherein one or more of steps (a) to (g) are performed in a closed, sterile system.

[00350] According to another embodiment, TALENs may be specifically designed, which allows higher rates of DSB events within the target cell(s) that are able to target a specific selection of genes. *See* U.S. Patent Publication No. 2013/0315884. The use of such rare cutting endonucleases increases the chances of obtaining double inactivation of target genes in transfected cells, allowing for the production of engineered cells, such as T-cells. Further, additional catalytic domains can be introduced with the TALEN to increase mutagenesis and enhance target gene inactivation. The TALENs described in U.S. Patent Publication No. 2013/0315884 were successfully used to engineer T-cells to make them suitable for immunotherapy. TALENs may also be used to inactivate various immune checkpoint genes in T-cells, including the inactivation of at least two genes in a single T-cell. *See* U.S. Patent Publication No. 2016/0120906. Additionally, TALENs may be used to inactivate genes encoding targets for immunosuppressive agents and T-cell receptors, as disclosed in U.S. Patent Publication No. 2018/0021379, which is incorporated by reference herein. Further, TALENs may be used to inhibit the expression of beta 2-microglobulin (B2M) and/or class II major histocompatibility complex transactivator (CIITA), as disclosed in U.S. Patent Publication No. 2019/0010514, which is incorporated by reference herein.

[00351] 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.

[00352] Non-limiting examples of TALE-nucleases targeting the PD-1 gene are provided in the following table. In these examples, the targeted genomic sequences contain two 17-base

pair (bp) long sequences (referred to as half targets, shown in upper case letters) separated by a 15-bp spacer (shown in lower case letters). Each half target is recognized by repeats of half TALE-nucleases listed in the table. Thus, according to particular embodiments, TALE-nucleases according to the invention recognize and cleave the target sequence selected from the group consisting of: SEQ ID NO: 127 and SEQ ID NO: 128. TALEN sequences and gene-editing methods are also described in Gautron, discussed above.

No.	Target PD-1 Sequence	Repeat Sequence	Half-TALE nuclease
1	TTCTCCCCAGCCCTGCT cgtggtgaccgaagg GGACAACGCCACCTTCA (SEQ ID NO:127)	Repeat PD-1-left (SEQ ID NO:129) Repeat PD-1-right (SEQ ID NO: 130)	PD-1-left TALEN (SEQ ID NO:133) PD-1-right TALEN (SEQ ID NO:134)
2	TACCTCTGTGGGGCCAT ctccctggcccccaa GGCGCAGATCAAAGAGA (SEQ ID NO:128)	Repeat PD-1-left (SEQ ID NO:131) Repeat PD-1-right (SEQ ID NO: 132)	PD-1-left TALEN (SEQ ID NO:135) PD-1-right TALEN (SEQ ID NO:136)

[00353] In an embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises the steps of:

(a) activating a first population of TILs obtained from a tumor resected from a patient using CD3 and CD28 activating beads or antibodies for 1 to 5 days;

(b) gene-editing at least a portion of the first population of TILs using electroporation of transcription activator-like effector nucleases targeting *PDCD1* in cytoporation medium to obtain a second population of TILs;

(c) optionally incubating the second population of TILs, wherein the incubation is performed at about 30-40 °C with about 5% CO_2;

(d) performing a first expansion by culturing the second population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a third 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 6 to 9 days to obtain the third population of TILs;

(e) performing a second expansion by supplementing the cell culture medium of the third population of TILs with additional IL-2, OKT-3, and antigen presenting cells

(APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 9 to 11 days to obtain the fourth population of TILs, wherein the fourth population of TILs is a therapeutic population of TILs;

(f) harvesting the therapeutic population of TILs obtained from step (e);

(g) 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; and

(h) wherein one or more of steps (a) to (g) are performed in a closed, sterile system.

[00354] In an embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises the steps of:

(a) activating a first population of TILs obtained from a tumor resected from a patient using CD3 and CD28 activating beads or antibodies for 1 to 5 days;

(b) gene-editing at least a portion of the first population of TILs using electroporation of transcription activator-like effector nucleases targeting SEQ ID NO:128 in cytoporation medium to obtain a second population of TILs;

(c) optionally incubating the second population of TILs, wherein the incubation is performed at about 30-40 °C with about 5% CO_2;

(d) performing a first expansion by culturing the second population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a third 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 6 to 9 days to obtain the third population of TILs;

(e) performing a second expansion by supplementing the cell culture medium of the third population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 9 to 11 days to obtain the fourth population of TILs, wherein the fourth population of TILs is a therapeutic population of TILs;

(f) harvesting the therapeutic population of TILs obtained from step (e);

(g) 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; and

(h) wherein one or more of steps (a) to (g) are performed in a closed, sterile system.

[00355] In an embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises the steps of:

(a) activating a first population of TILs obtained from a tumor resected from a patient

using CD3 and CD28 activating beads or antibodies for 1 to 5 days;

(b) gene-editing at least a portion of the first population of TILs using electroporation of transcription activator-like effector nuclease mRNA according to SEQ ID NO:135 and SEQ ID NO:136 in cytoporation medium to obtain a second population of TILs;

(c) optionally incubating the second population of TILs, wherein the incubation is performed at about 30-40 °C with about 5% CO_2;

(d) performing a first expansion by culturing the second population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a third 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 6 to 9 days to obtain the third population of TILs;

(e) performing a second expansion by supplementing the cell culture medium of the third population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 9 to 11 days to obtain the fourth population of TILs, wherein the fourth population of TILs is a therapeutic population of TILs;

(f) harvesting the therapeutic population of TILs obtained from step (e);

(g) 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; and

(h) wherein one or more of steps (a) to (g) are performed in a closed, sterile system.

[00356] Other 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, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00357] 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. These disclosed examples include the use of a non-naturally occurring DNA-binding polypeptide that has two or more TALE-repeat units containing a repeat RVD, an N-cap polypeptide made of residues of a TALE protein, and a C-cap polypeptide made of a fragment of a full length C-terminus region of a TALE protein.

[00358] Examples of TALEN designs and design strategies, activity assessments, screening strategies, and methods that can be used to efficiently perform TALEN-mediated gene integration and inactivation, and which may be used in accordance with embodiments of the present invention, are described in Valton, *et al.*, *Methods*, **2014**, 69, 151-170, which is incorporated by reference herein.

[00359] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without

opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a TALE nuclease system for modulating the expression of at least one protein.

[00360] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a TALE nuclease system for suppressing the expression of PD-1 and LAG-3.

3. Zinc Finger Methods

[00361] 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 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.

[00362] 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.

[00363] 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).

[00364] 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.

[00365] 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, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00366] 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.

[00367] 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.

[00368] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without

opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a zinc finger nuclease system for modulating the expression of at least one protein.

[00369] According to one embodiment, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising a 4-1BB agonist antibody for about 2 to 5 days;

(d) adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a zinc finger nuclease system for suppressing the expression of PD-1 and LAG-3.

IV. TIL Manufacturing Processes

[00370] An exemplary TIL process known as process 2A containing some of these features is depicted in Figure 1, and some of the advantages of this embodiment of the present invention over process 1C are described in Figure 2, as does Figure 14. Process 1C is shown for comparison in Figure 3. Two alternative timelines for TIL therapy based on process 2A are shown in Figure 4 (higher cell counts) and Figure 5 (lower cell counts). An embodiment of process 2A is shown in Figure 6 as well as Figure 9. Figures 13 and 14 further provides an exemplary 2A process compared to an exemplary 1C process.

[00371] 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.

[00372] 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.

[00373] In some embodiments, the first expansion (including processes referred to as the preREP as well as processes shown in Figure 9 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 9 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 9) is shortened to 11 days and the second expansion (for example, an expansion as described in Step D in Figure 9) is shortened to 11 days, as discussed in the Examples and shown in Figures 4, 5 and 27. In some embodiments, the combination of the first expansion and second expansion (for example, expansions described as Step B and Step D in Figure 9) is shortened to 22 days, as discussed in detail below and in the examples and figures.

[00374] The "Step" Designations A, B, C, *etc.*, below are in reference to Figure 9 and in reference to certain embodiments described herein. The ordering of the Steps below and in Figure 9 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

[00375] 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.

[00376] 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, useful TILs are obtained from malignant melanoma tumors, as these have been reported to have particularly high levels of TILs.

[00377] 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, but are not limited to, sarcomas, carcinomas, and lymphomas, such as cancers of the lung, breast, triple negative breast cancer, prostate, colon, rectum, and bladder. 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, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, and non-small cell lung carcinoma. 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.

[00378] The term "hematological malignancy" refers 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.

[00379] 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 gentamicin, 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.

[00380] In general, the harvested cell suspension is called a "primary cell population" or a "freshly harvested" cell population.

[00381] 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.

[00382] 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 9). 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.

[00383] 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.

[00384] 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.

[00385] 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.

[00386] 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 9.

B. STEP B: First Expansion

1. Young TILs

[00387] 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.

[00388] 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 9. 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 13. 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).

[00389] After dissection or digestion of tumor fragments, for example such as described in Step A of Figure 9, 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 1×10^8 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 1×10^8 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 1×10^8 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 1×10^8 bulk TIL cells.

[00390] 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 9, 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.

[00391] 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 1×10^6 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.

[00392] 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.

[00393] 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 1×10^8 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 $20\text{-}30\times10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 20×10^6 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 $4\text{-}8\times10^6$ 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 6×10^6 IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepare as described in Example 4. 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.

[00394] 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.

[00395] 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.

[00396] In an embodiment, the cell culture medium comprises OKT-3 antibody. The OKT-3 antibody may be present in the cell culture medium beginning on day 0 of the REP (*i.e.*, the start day of the REP) and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). 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.

[00397] 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 10–40x 10^6 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 5. 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.

[00398] In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 9, 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 9, 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 9. In some embodiments, the first expansion of Step B is shortened to 10-14 days, as discussed in the Examples and shown in Figures 4 and 5. In some embodiments, the first expansion is shortened to 11 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 9.

[00399] 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.

[00400] 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 9, 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 9 and as described herein.

[00401] In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 9) 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 to14 days, as discussed in the Examples and shown in Figures 4 and 5. In some embodiments, the first expansion of Step B is shortened to 10 to14 days, as discussed in the Examples and shown in Figures 4, 5, and 9. In some embodiments, the first expansion is shortened to 11 days, as discussed in the Examples and shown in Figures 4, 5, and 9.

[00402] In some embodiments, the first expansion, for example, Step B according to Figure 9, 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.

[00403] In some embodiments, the first expansion is performed using an additional 4-1BB agonist antibody added to the cell culture medium at the start of the expansion, using any of the 4-1BB agonist antibodies described hereinafter.

C. STEP C: First Expansion to Second Expansion Transition

[00404] 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 9, 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.

[00405] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 9) 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 9) 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.

[00406] 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.

[00407] 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 9). 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.

[00408] In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 9, 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.

D. STEP D: Second Expansion

[00409] 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 9). 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 9). 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.

[00410] 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 9) 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.

[00411] 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 9). 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.

[00412] In an embodiment, the cell culture medium further comprises IL-2. In a 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.

[00413] In an embodiment, the cell culture medium comprises OKT3 antibody. The OKT-3 antibody may be present in the cell culture medium beginning on day 0 of the REP (*i.e.*, the start day of the REP) and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT3 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 OKT3 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 OKT3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody.

[00414] 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 9, 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 9 and as described herein.

[00415] In some embodiments, the second expansion can be conducted in a supplemented cell culture medium comprising IL-2, OKT-3, and antigen-presenting feeder cells. 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).

[00416] 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.

[00417] 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.

[00418] 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.

[00419] 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.

[00420] 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.

[00421] 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.

[00422] 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 9) 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.

[00423] 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.

[00424] 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.

[00425] 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 Cellometer K2 Image Cytometer Automatic Cell Counter protocol described, for example, in Example 15.

[00426] 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.

[00427] 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 5x10^6 or 10x10^6 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.

[00428] 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).

[00429] 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.

[00430] In some embodiments, the second expansion, for example, Step D according to Figure 9, 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

[00431] In an embodiment, the second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 9, 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.

[00432] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, in particular example 14, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

[00433] 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). See, for example, Example 14.

[00434] 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. See, for example, Example 13.

[00435] 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.

[00436] 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.

[00437] 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.

[00438] 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.

[00439] 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 Figures 4, 5, and 9.

[00440] In an embodiment, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

[00441] 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.

[00442] 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.

3. Anti-CD3 Antibodies

[00443] In some embodiments, the culture media used in expansion methods described herein (including those referred to as REP, see for example, Figure 9) 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.

[00444] 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).

[00445] In some embodiments, the anti-CD3 antibody, such as OKT3, may be added to TIL culture 2 days, 3 days, 4 days, or 5 days prior to an electroporation step. In some embodiments, the anti-CD3 antibody, such as OKT3, may be added immediately before an electroporation step. In some embodiments, the anti-CD3 antibody, such as OKT3, may be added immediately after an electroporation step. In some embodiments, the anti-CD3 antibody, such as OKT3, may be added 2 days, 3 days, 4 days, or 5 days after an electroporation step.

4. 4-1BB and OX40 Agonists

[00446] According to an embodiment, the cell culture medium further comprises a 4-1BB (CD137) agonist and/or an OX40 agonist during the first expansion, the second expansion, or both. The gene-editing may be carried out after the 4-1BB agonist and/or the OX40 agonist are introduced into the cell culture medium. Alternatively, the gene-editing may be carried out before the 4-1BB agonist and/or the OX40 agonist are introduced into the cell culture medium.

[00447] 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.

[00448] 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.

[00449] 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.

[00450] 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 3.

TABLE 3. 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 TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC 120 CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE 180 PGHSPQIISF FLALTSTALL FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG 240 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 CNICRVCAGY FRFKKFCSST HNAECECIEG FHCLGPQCTR CEKDCRPGQE LTKQGCKTCS 120 LGTFNDQNGT GVCRPWTNCS LDGRSVLKTG TTEKDVVCGP PVVSFSPSTT ISVTPEGGPG 180 GHSLQVLTLF LALTSALLLA LIFITLLFSV LKWIRKKFPH IFKQPFKKTT GAAQEEDACS 240 CRCPQEEEGG GGGYEL 256

[00451] 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.

[00452] 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 7.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 8×10^5 l/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human or murine 4-1BB with a k_{assoc} of about 1×10^6 1/M·s or faster.

[00453] 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 2×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower , binds to human or murine 4-1BB with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human or murine 4-1BB with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human or murine 4-1BB with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[00454] 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.

[00455] 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 4. 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.

[00456] 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.

[00457] 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.

[00458] 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.

[00459] 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 4. 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 SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCARGY GIFDYWGQGT LVTVSSASTK GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVHTFP AVLQSSGLYS LSSVVTVPSS NFGTQTYTCN VDHKPSNTKV DKTVERKCCV ECPPCPAPPV AGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTFRVVSV LTVVHQDWLN GKEYKCKVSN KGLPAPIEKT ISKTKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PMLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP G	60 120 180 240 300 360 420 441
SEQ ID NO:12 light chain for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVLGQ PKAAPSVTLF PPSSEELQAN KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTPSKQS NNKYAASSYL SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS	60 120 180 214
SEQ ID NO:13 heavy chain variable region for utomilumab	EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMG KIYPGDSYTN YSPSFQGQVT ISADKSISTA YLQWSSLKAS DTAMYYCARG YGIFDYWGQ GTLVTVSS	60 118
SEQ ID NO:14 light chain variable region for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVL	60 108
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

[00460] 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 5. 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.

[00461] 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.

[00462] 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.

[00463] 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.

[00464] 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 5. 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 PSLESRVTIS VDTSKNQFSL KLSSVTAADT AVYYCARDYG PGNYDWYFDL WGRGTLVTVS SASTKGPSVF PLAPCSRSTS ESTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTK TYTCNVDHKP SNTKVDKRVE SKYGPPCPPC PAPEFLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK	60 120 180 240 300 360 420 448
SEQ ID NO:22 light chain for urelumab	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPALTF CGGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC	60 120 180 216
SEQ ID NO:23 variable heavy chain for urelumab	MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YYWSWIRQSP EKGLEWIGEI NHGGYVTYNP SLESRVTISV DTSKNQFSLK LSSVTAADTA VYYCARDYGP	60 120
SEQ ID NO:24 variable light chain for urelumab	MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ	60 110
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

[00465] 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.

[00466] 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.

[00467] 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) of Figure 22, or a fragment, derivative, conjugate, variant, or biosimilar thereof.

[00468] In structures I-A and I-B of Figure 22, 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 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.

[00469] Amino acid sequences for the other polypeptide domains of structure I-A are given in Table 6. 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 6. 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 Fc domain	KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW 60 YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS 120 KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV 180 LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK 230
SEQ ID NO:32 linker	GGPGSSKSCD KTHTCPPCPA PE 22
SEQ ID NO:33 linker	GGSGSSKSCD KTHTCPPCPA PE 22
SEQ ID NO:34 linker	GGPGSSSSSS SKSCDKTHTC PPCPAPE 27
SEQ ID NO:35 linker	GGSGSSSSSS SKSCDKTHTC PPCPAPE 27
SEQ ID NO:36 linker	GGPGSSSSSS SSSKSCDKTH TCPPCPAPE 29

SEQ ID NO:37 linker	GGSGSSSSSS SSSKSCDKTH TCPPCPAPE	29
SEQ ID NO:38 linker	GGPGSSGSGS SDKTHTCPPC PAPE	24
SEQ ID NO:39 linker	GGPGSSGSGS DKTHTCPPCP APE	23
SEQ ID NO:40 linker	GGPSSSGSDK THTCPPCPAP E	21
SEQ ID NO:41 linker	GGSSSSSSSS GSDKTHTCPP CPAPE	25

[00470] Amino acid sequences for the other polypeptide domains of structure I-B are given in Table 7. 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 SEQ ID NO:43 to SEQ ID NO:45.

TABLE 7. 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 Fc domain	METDTLLLWV LLLWVPAGNG DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG	60 120 180 240 246
SEQ ID NO:43 linker	SGSGSGSGSG S	11
SEQ ID NO:44 linker	SSSSSSGSGS GS	12
SEQ ID NO:45 linker	SSSSSSGSGS GSGSGS	16

[00471] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B of Figure 22 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 8, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[00472] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B of Figure 22 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.

[00473] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B of Figure 22 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 8, wherein the V_H and V_L domains are connected by a linker.

TABLE 8. 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 SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAALA LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ GATVLGLFRV TPEIPAGLPS PRSE	60 120 180 240 254
SEQ ID NO:47 4-1BBL soluble domain	LRQGMFAQLV AQNVLLIDGP LSWYSDPGLA GVSLTGGLSY KEDTKELVVA KAGVYYVFFQ LELRRVVAGE GSGSVSLALH LQPLRSAAGA AALALTVDLP PASSEARNSA FGFQGRLLHL SAGQRLGVHL HTEARARHAW QLTQGATVLG LFRVTPEIPA GLPSPRSE	60 120 168
SEQ ID NO:48 variable heavy chain for 4B4-1-1 version 1	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVS	60 118
SEQ ID NO:49 variable light chain for 4B4-1-1 version 1	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIK	60 107
SEQ ID NO:50 variable heavy chain for 4B4-1-1 version 2	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVSA	60 119
SEQ ID NO:51 variable light chain for 4B4-1-1 version 2	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIKR	60 108
SEQ ID NO:52 variable heavy chain for H39E3-2	MDWTWRILFL VAAATGAHSE VQLVESGGGL VQPGGSLRLS CAASGFTFSD YWMSWVRQAP GKGLEWVADI KNDGSYTNYA PSLTNRFTIS RDNAKNSLYL QMNSLRAEDT AVYYCARELT	60 120
SEQ ID NO:53 variable light chain for H39E3-2	MEAPAQLLFL LLLWLPDTTG DIVMTQSPDS LAVSLGERAT INCKSSQSLL SSGNQKNYL WYQQKPGQPP KLLIYYASTR QSGVPDRFSG SGSGTDFTLT ISSLQAEDVA	60 110

[00474] 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.

[00475] 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.

[00476] 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.

[00477] 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.

[00478] The OX40 (CD134) 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).

[00479] 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.

[00480] 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.

[00481] 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 9.

TABLE 9. Amino acid sequences of OX40 antigens.

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

[00482] 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.

[00483] 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 7.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human or murine OX40 with a k_{assoc} of about 1×10^6 1/M·s or faster.

[00484] 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 2×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower , binds to human or murine OX40 with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human or murine OX40 with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human or murine OX40 with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[00485] 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.

[00486] 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 10. 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.

[00487] 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.

[00488] 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.

[00489] 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.

[00490] 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 10. 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 PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVTVS SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K	60 120 180 240 300 360 420 451
SEQ ID NO:57 light chain for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	60 120 180 214
SEQ ID NO:58 heavy chain variable region for tavolixizumab	QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVT	60 118
SEQ ID NO:59 light chain variable region for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKR	60 108
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

[00491] 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 11.

[00492] 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.

[00493] 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.

[00494] 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.

[00495] 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 11. 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 ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSSAS TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSNFGTQTYT CNVDHKPSNT KVDKTVERKC CVECPPCPAP PVAGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTFRVV SVLTVVHQDW LNGKEYKCKV SNKGLPAPIE KTISKTKGQP REPQVYTLPP SREEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPMLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK	60 120 180 240 300 360 420 444
SEQ ID NO:67 light chain for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	60 120 180 214
SEQ ID NO:68 heavy chain variable region for 11D4	EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSS	60 118
SEQ ID NO:69 light chain variable region for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIK	60 107
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

[00496] 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 12.

[00497] 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.

[00498] 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.

[00499] 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.

[00500] 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 12. 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 ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV TVSSASTKGP SVFPLAPCSR STSESTAALG CLVKDYFPEP VTVSWNSGAL TSGVHTFPAV LQSSGLYSLS SVVTVPSSNF GTQTYTCNVD HKPSNTKVDK TVERKCCVEC PPCPAPPVAG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVQFNW YVDGVEVHNA KTKPREEQFN STFRVVSVLT VVHQDWLNGK EYKCKVSNKG LPAPIEKTIS KTKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPM LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:77 light chain for 18D8	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC	60 120 180 213
SEQ ID NO:78 heavy chain variable region for 18D8	EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV TVSS	60 120 124
SEQ ID NO:79 light chain variable region for 18D8	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIK	60 106

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

[00501] 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 13.

[00502] 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.

[00503] 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.

[00504] 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 13. 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 PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS	60 120
SEQ ID NO:87 light chain variable region for Hu119-122	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K	60 111
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

[00505] 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 14.

[00506] 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.

[00507] 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.

[00508] 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 14. 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 ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV SS	60 120 122
SEQ ID NO:95 light chain variable region for Hu106-222	DIQMTQSPSS LSASVGDRVT ITCKASQDVS TAVAWYQQKP GKAPKLLIYS ASYLYTGVPS RFSGSGSGTD FTFTISSLQP EDIATYYCQQ HYSTPRTFGQ GTKLEIK	60 107
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

[00509] 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.

[00510] 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.

[00511] 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.

[00512] 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 6. 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 7. 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.

[00513] 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 15, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[00514] 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.

[00515] 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 15, wherein the V_H and V_L domains are connected by a linker.

TABLE 15. 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 OX40L	MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL QVSHRYPRIQ SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYFS QEVNISLHYQ KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF CVL	60 120 180 183

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

antibody	
SEQ ID NO:121 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGESLKL SCESNEYEFP SHDMSWVRKT PEKRLELVAA INSDGGSTYY 60 PDTMERRFII SRDNTKKTLY LQMSSLRSED TALYYCARHY DDYYAWFAYW GQGTLVTVSA 120
SEQ ID NO:122 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY 60 PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS 120
SEQ ID NO:123 light chain variable region of humanized antibody	DIVLTQSPAS LAVSLGQRAT ISCRASKSVS TSGYSYMHWY QQKPGQPPKL LIYLASNLES 60 GVPARFSGSG SGTDFTLNIH PVEEEDAATY YCQHSRELPL TFGAGTKLEL K 111
SEQ ID NO:124 light chain variable region of humanized antibody	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES 60 GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K 111
SEQ ID NO:125 heavy chain variable region	MYLGLNYVFI VFLLNGVQSE VKLEESGGGL VQPGGSMKLS CAASGFTFSD AWMDWVRQSP 60 EKGLEWVAEI RSKANNHATY YAESVNGRFT ISRDDSKSSV YLQMNSLRAE DTGIYYCTWG 120 EVFYFDYWGQ GTTLTVSS 138
SEQ ID NO:126 light chain variable region	MRPSIQFLGL LLFWLHGAQC DIQMTQSPSS LSASLGGKVT ITCKSSQDIN KYIAWYQHKP 60 GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLLTFGAG 120 TKLELK 126

[00516] 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.

[00517] 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.

[00518] 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.

[00519] 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.

[00520] In an embodiment, the OX40 agonist is Creative Biolabs OX40 agonist monoclonal antibody MOM-18455, commercially available from Creative Biolabs, Inc., Shirley, NY, USA.

[00521] In an embodiment, the OX40 agonist is OX40 agonistic antibody clone Ber-ACT35 commercially available from BioLegend, Inc., San Diego, CA, USA.

E. STEP E: Harvest TILS

[00522] 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 9. In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 9.

[00523] 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.

[00524] 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.

[00525] In some embodiments, the harvest, for example, Step E according to Figure 9, 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.

F. STEP F: Final Formulation and Transfer to Infusion Bag

[00526] After Steps A through E as provided in an exemplary order in Figure 9 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.

[00527] 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. Pharmaceutical Compositions, Dosages, and Dosing Regimens

[00528] 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.

[00529] Any suitable dose of TILs can be administered. In some embodiments, from about 2.3×10^{10} to about 13.7×10^{10} TILs are administered, with an average of around 7.8×10^{10} TILs, particularly if the cancer is melanoma. In an embodiment, about 1.2×10^{10} to about 4.3×10^{10} of TILs are administered. In some embodiments, about 3×10^{10} to about 12×10^{10} TILs are administered. In some embodiments, about 4×10^{10} to about 10×10^{10} TILs are administered. In some embodiments, about 5×10^{10} to about 8×10^{10} TILs are administered. In some embodiments, about 6×10^{10} to about 8×10^{10} TILs are administered. In some embodiments, about 7×10^{10} to about 8×10^{10} TILs are administered. In some embodiments, the therapeutically effective dosage is about 2.3×10^{10} to about 13.7×10^{10}. In some embodiments, the therapeutically effective dosage is about 7.8×10^{10} TILs, particularly of the cancer is melanoma. In some embodiments, the therapeutically effective dosage is about 1.2×10^{10} to about 4.3×10^{10} of TILs. In some embodiments, the therapeutically effective dosage is about 3×10^{10} to about 12×10^{10} TILs. In some embodiments, the therapeutically effective dosage is about 4×10^{10} to about 10×10^{10} TILs. In some embodiments, the therapeutically effective dosage is about 5×10^{10} to about 8×10^{10} TILs. In some embodiments, the therapeutically effective dosage is about 6×10^{10} to about 8×10^{10} TILs. In some embodiments, the therapeutically effective dosage is about 7×10^{10} to about 8×10^{10} TILs.

[00530] In some embodiments, the number of the TILs provided in the pharmaceutical compositions of the invention is about 1×10^{6}, 2×10^{6}, 3×10^{6}, 4×10^{6}, 5×10^{6}, 6×10^{6}, 7×10^{6}, 8×10^{6}, 9×10^{6}, 1×10^{7}, 2×10^{7}, 3×10^{7}, 4×10^{7}, 5×10^{7}, 6×10^{7}, 7×10^{7}, 8×10^{7}, 9×10^{7}, 1×10^{8}, 2×10^{8}, 3×10^{8}, 4×10^{8}, 5×10^{8}, 6×10^{8}, 7×10^{8}, 8×10^{8}, 9×10^{8}, 1×10^{9}, 2×10^{9}, 3×10^{9}, 4×10^{9}, 5×10^{9}, 6×10^{9}, 7×10^{9}, 8×10^{9}, 9×10^{9}, 1×10^{10}, 2×10^{10}, 3×10^{10}, 4×10^{10}, 5×10^{10}, 6×10^{10}, 7×10^{10}, 8×10^{10}, 9×10^{10}, 1×10^{11}, 2×10^{11}, 3×10^{11}, 4×10^{11}, 5×10^{11}, 6×10^{11}, 7×10^{11}, 8×10^{11}, 9×10^{11}, 1×10^{12}, 2×10^{12}, 3×10^{12}, 4×10^{12}, 5×10^{12}, 6×10^{12}, 7×10^{12}, 8×10^{12}, 9×10^{12}, 1×10^{13}, 2×10^{13}, 3×10^{13}, 4×10^{13}, 5×10^{13}, 6×10^{13}, 7×10^{13}, 8×10^{13}, and 9×10^{13}. In an embodiment, the number of the TILs provided in the pharmaceutical compositions of the invention is in the range of 1×10^{6} to 5×10^{6}, 5×10^{6} to 1×10^{7}, 1×10^{7} to 5×10^{7}, 5×10^{7} to 1×10^{8}, 1×10^{8} to 5×10^{8}, 5×10^{8} to 1×10^{9}, 1×10^{9} to 5×10^{9}, 5×10^{9} to 1×10^{10}, 1×10^{10} to 5×10^{10}, 5×10^{10} to 1×10^{11}, 5×10^{11} to 1×10^{12}, 1×10^{12} to 5×10^{12}, and 5×10^{12} to 1×10^{13}.

[00531] 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.

[00532] 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.

[00533] 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.

[00534] 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.

[00535] 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.

[00536] 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.

[00537] 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.

[00538] 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.

[00539] In some embodiments, an effective dosage of TILs is about 1×10^6, 2×10^6, 3×10^6, 4×10^6, 5×10^6, 6×10^6, 7×10^6, 8×10^6, 9×10^6, 1×10^7, 2×10^7, 3×10^7, 4×10^7, 5×10^7, 6×10^7, 7×10^7, 8×10^7, 9×10^7, 1×10^8, 2×10^8, 3×10^8, 4×10^8, 5×10^8, 6×10^8, 7×10^8, 8×10^8, 9×10^8, 1×10^9, 2×10^9, 3×10^9, 4×10^9, 5×10^9, 6×10^9, 7×10^9, 8×10^9, 9×10^9, 1×10^{10}, 2×10^{10}, 3×10^{10}, 4×10^{10}, 5×10^{10}, 6×10^{10}, 7×10^{10}, 8×10^{10}, 9×10^{10}, 1×10^{11}, 2×10^{11}, 3×10^{11}, 4×10^{11}, 5×10^{11}, 6×10^{11}, 7×10^{11}, 8×10^{11}, 9×10^{11}, 1×10^{12}, 2×10^{12}, 3×10^{12}, 4×10^{12}, 5×10^{12}, 6×10^{12}, 7×10^{12}, 8×10^{12}, 9×10^{12}, 1×10^{13}, 2×10^{13}, 3×10^{13}, 4×10^{13}, 5×10^{13}, 6×10^{13}, 7×10^{13}, 8×10^{13}, and 9×10^{13}. In some embodiments, an effective dosage of TILs is in the range of 1×10^6 to 5×10^6, 5×10^6 to 1×10^7, 1×10^7 to 5×10^7, 5×10^7 to 1×10^8, 1×10^8 to 5×10^8, 5×10^8 to 1×10^9, 1×10^9 to 5×10^9, 5×10^9 to 1×10^{10}, 1×10^{10} to 5×10^{10}, 5×10^{10} to 1×10^{11}, 5×10^{11} to 1×10^{12}, 1×10^{12} to 5×10^{12}, and 5×10^{12} to 1×10^{13}.

[00540] 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.

[00541] 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.

[00542] 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.

H. Cryopreservation of TILs

[00543] As discussed above, and exemplified in Steps A through E as provided in Figure 9, 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 9) 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 8 and 9.

[00544] 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.

I. Closed Systems for TIL Manufacturing

[00545] 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.

[00546] 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.

[00547] As provided on the FDA website, closed systems with sterile methods are known and well described. See, https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076779.htm, as referenced above and provided in pertinent part below.

[00548] 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. This guidance describes recommended practices and procedures for use of these devices. This guidance does not address the data or information that a manufacturer of a sterile connecting device must submit to FDA in order to obtain approval or clearance for marketing. It is also important to note that the use of an approved or cleared sterile connecting device for purposes not authorized in the labeling may cause the device to be considered adulterated and misbranded under the Federal Food, Drug and Cosmetic Act.

[00549] 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.

[00550] 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%.

[00551] The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

[00552] 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.

[00553] 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.

[00554] 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.

C. Cell Cultures

[00555] In an embodiment, a method for expanding TILs, including those discuss above as well as exemplified in Figure 9, 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.

[00556] 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).

[00557] 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.

[00558] 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.

[00559] 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.

D. Optional Cryopreservation of TILs

[00560] Either the bulk TIL population or the expanded 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 9. 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.

[00561] 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.

[00562] 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.

[00563] According to particular embodiments, a cryopreservation composition (also referred to herein as a "dimethylsulfoxide-based cryopreservation medium") comprises a population of TILs prepared in accordance with the present invention (e.g., in an amount of 1 X 10^6 to 9 X 10^{13}), a cryoprotectant medium comprising DMSO that is suitable for preserving cells in low-temperature environments such as -70°C to -196°C (e.g., CryoStor® CS10) and an electrolyte solution (e.g., an isotonic solution such as PlasmaLyte® A). In a preferred embodiment, the cryoprotectant medium and electrolyte solution are present in a ratio of between about 1.2:1 and about 1:1.2, or between about 1.1:1 and about 1:1.1, or preferably about 1:1. According to one embodiment, the electrolyte solution comprises one or more of sodium, potassium, magnesium, acetate, chloride and gluconate, or a combination thereof; for example, the electrolyte solution may comprise sodium chloride, sodium gluconate, sodium acetate trihydrate, potassium chloride and magnesium chloride. According to an embodiment, the electrolyte solution has a pH between about 7 and about 8, preferably between about 7.2 and about 7.6, or about 7.4. Preferably, the cryopreservation composition further comprises one or more stabilizers (e.g., human serum albumin) and/or one or more

lymphocyte growth factors (e.g., IL-2). For example, each of the cryoprotectant medium and the electrolyte solution may be present in the cryopreservation composition in an amount of about 20 mL to about 100 mL, or about 30 mL to about 70 mL, or about 40 mL to about 60 mL, or about 50 mL; human serum albumin may be present in an amount of about 0.01 g to about 2.0 g, or about 0.1 g to about 1.0 g, or about 0.5 g; and IL-2 may be present in an amount of about 0.001 mg to about 0.005 mg, or about 0.0015 mg to about 0.0025 mg, or about 0.0018 mg. The cryopreservation medium may optionally comprise one or more additional additives or excipients, such as pH adjusters, preservatives, etc.

[00564] According to one embodiment, a cryopreservation composition containing a population of TILs is composed of the following:

Nominal Composition (%v/V) of Drug Product (100 mL basis)

Ingredient	Nominal Quantity per 100 mL fill	Function
Tumor Infiltrating Lymphocytes	83×10^7 to 3.0×10^{10}	Active Ingredient
CryoStor® CS10*	50 mL	Cryopreservation medium
PlasmaLyte A	50 mL	Isotonic agent
Human Serum Albumin (HSA)	0.5 g	Stabilizer
Interleukin-2	0.0018 mg (30,000 IU)	Lymphocyte growth factor
Total	100 mL	

* Cryostor CS10 contains 10% dimethylsulfoxide (DMSO)

[00565] 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.

[00566] 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.

[00567] 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.

V. Methods of Treating Cancers and Other Diseases

[00568] The compositions and methods described herein can be used in a method for treating diseases. In an embodiment, they are for use in treating hyperproliferative disorders. They may also be used in treating other disorders as described herein and in the following paragraphs.

[00569] In some embodiments, the hyperproliferative disorder is cancer. In some embodiments, the hyperproliferative disorder is a solid tumor cancer. In some embodiments, the solid tumor 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)), renal cancer, and renal cell carcinoma. In some embodiments, the hyperproliferative disorder is a hematological malignancy. In some embodiments, the solid tumor cancer is selected from the group consisting of chronic lymphocytic leukemia, acute lymphoblastic leukemia, diffuse large B cell lymphoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, follicular lymphoma, and mantle cell lymphoma.

[00570] 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 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 intravenously at 720,000 IU/kg every 8 hours to physiologic tolerance.

[00571] Efficacy of the TILs described herein in treating, preventing and/or managing the indicated diseases or disorders can be tested using various models known in the art, which provide guidance for treatment of human disease. For example, models for determining

efficacy of treatments for ovarian cancer are described, *e.g.*, in Mullany, *et al.*, *Endocrinology* **2012,** *153,* 1585-92; and Fong, *et al.*, *J. Ovarian Res.* **2009,** *2,* 12. Models for determining efficacy of treatments for pancreatic cancer are described in Herreros-Villanueva, *et al.*, *World J. Gastroenterol.* **2012,** *18,* 1286-1294. Models for determining efficacy of treatments for breast cancer are described, *e.g.*, in Fantozzi, *Breast Cancer Res.* **2006,** *8,* 212. Models for determining efficacy of treatments for melanoma are described, *e.g.*, in Damsky, *et al.*, *Pigment Cell & Melanoma Res.* **2010,** *23,* 853–859. Models for determining efficacy of treatments for lung cancer are described, *e.g.*, in Meuwissen, *et al.*, *Genes & Development*, **2005,** *19,* 643-664. Models for determining efficacy of treatments for lung cancer are described, *e.g.*, in Kim, *Clin. Exp. Otorhinolaryngol.* **2009,** *2,* 55-60; and Sano, *Head Neck Oncol.* **2009,** *1,* 32.

[00572] In some embodiments, IFN-gamma (IFN-γ) is indicative of treatment efficacy for hyperproliferative disorder treatment. 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 of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 20 or Figure 21. In some embodiments, the TILs obtained by the present method provide for increased IFN-γ in the blood of subjects treated with the TILs of the present method as compared to subjects treated with TILs prepared using methods referred to as process 1C, as exemplified in Figure 13. 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 20 or Figure 21. 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 20 or Figure 21. 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 20 or Figure 21. 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 20 or Figure 21. 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 20 or Figure 21. 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 20 or Figure 21. In some embodiments, IFN-γ is measured using a Quantikine ELISA kit. In some embodiments, IFN-γ is measured using a Quantikine ELISA kit. In some embodiments, IFN-γ is measured in TILs *ex vivo* from a patient treated with the TILs produced by the methods of the present invention. In some embodiments, IFN-γ is measured in blood in a patient treated with the TILs produced by the methods of the present invention. In some embodiments, IFN-γ is measured in serum in a patient treated with the TILs produced by the methods of the present invention.

[00573] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 20 or Figure 21, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 20 or Figure 21, 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 20 or Figure 21. 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 9. 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 9. 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 9. 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 9. 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 9. 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 9.

1. **Methods of co-administration**

[00574] In some embodiments, the TILs produced as described herein, including for example TILs derived from a method described in Figure 20 or Figure 21, can be administered in combination with one or more immune checkpoint regulators, such as the antibodies described below. For example, antibodies that target PD-1 and which can be co-administered with the TILs of the present invention include, *e.g.*, but are 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 immune response, are suitable for use in co-administration methods with TILs produced according to Steps A through F as described herein. 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-Ll 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 suitable for use in co-administration methods with TILs produced according to Steps A through F as described herein. In some embodiments, the subject administered the combination of TILs produced according to Steps A through F is co administered with a and anti-PD-1 antibody when the patient has a cancer type that is refractory to administration of the anti-PD-1 antibody alone. In some embodiments, the patient is administered TILs in combination with and anti-PD-1 when the patient has refractory melanoma. In some embodiments, the patient is administered TILs in combination with and anti-PD-1 when the patient has non-small-cell lung carcinoma (NSCLC).

2. Optional Lymphodepletion Preconditioning of Patients

[00575] 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.

[00576] 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.

[00577] 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.

[00578] In some embodiments, the fludarabine is administered at a concentration of 0.5 μg/mL to 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.

[00579] 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.

[00580] 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.

[00581] 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

[00582] 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 the 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.

[00583] 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 18×10^6 IU/m^2 administered intravenously over 6 hours, followed by 18×10^6 IU/m^2 administered intravenously over 12 hours, followed by 18×10^6 IU/m^2 administered intravenously over 24 hrs, followed by 4.5×10^6 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.

[00584] 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. Adoptive Cell Transfer

[00585] Adoptive cell transfer (ACT) is a very effective form of immunotherapy and involves the transfer of immune cells with antitumor activity into cancer patients. ACT is a

treatment approach that involves the identification, *in vitro*, of lymphocytes with antitumor activity, the in vitro expansion of these cells to large numbers and their infusion into the cancer-bearing host. Lymphocytes used for adoptive transfer can be derived from the stroma of resected tumors (tumor infiltrating lymphocytes or TILs). TILs for ACT can be prepared as described herein. In some embodiments, the TILs are prepared, for example, according to a method as described in Figure 9. They can also be derived or from blood if they are genetically engineered to express antitumor T-cell receptors (TCRs) or chimeric antigen receptors (CARs), enriched with mixed lymphocyte tumor cell cultures (MLTCs), or cloned using autologous antigen presenting cells and tumor derived peptides. ACT in which the lymphocytes originate from the cancer-bearing host to be infused is termed autologous ACT. U.S. Publication No. 2011/0052530 relates to a method for performing adoptive cell therapy to promote cancer regression, primarily for treatment of patients suffering from metastatic melanoma, which is incorporated by reference in its entirety for these methods. In some embodiments, TILs can be administered as described herein. In some embodiments, TILs can be administered in a single dose. Such administration may be by injection, *e.g.*, intravenous injection. In some embodiments, TILs and/or cytotoxic lymphocytes 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 and/or cytotoxic lymphocytes may continue as long as necessary.

EXAMPLES

[00586] 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. Production of a Cryopreserved TIL Therapy.

[00587] This example describes the cGMP manufacture of TIL therapy in G-Rex Flasks according to current Good Tissue Practices and current Good Manufacturing Practices.

PROCESS REFERENCE Expansion Plan

Estimated Day (post-seed)	Activity	Target Criteria	Anticipated Vessels	Estimated Total Volume (mL)
0	Tumor Dissection	≤ 50 desirable tumor fragments per G-Rex100MCS	G-Rex100MCS 1 flask	≤1000
11	REP Seed	5 – 200 x 10^6 viable cells per G-Rex500MCS	G-Rex500MCS 1 flasks	≤5000
16	REP Split	1 x 10^9 viable cells per G-Rex500MCS	G-Rex500MCS ≤5 flasks	≤25000
22	Harvest	Total available cells	3-4 CS-750 bags	≤530

Flask Volumes:

Flask Type	Working Volume/Flask (mL)
G-Rex100MCS	1000
G-Rex500MCS	5000

EQUIPMENT

[00588] Equipment List: Day 0 CM1 Media Preparation /Tumor Wash Preparation/Tumor Dissection:

- Magnehelic Gauge
- Biological Safety Cabinet (BSC)
- Incubator
- CO2 Analyzer
- Micropipetter (100-1000µL)
- Pipet-Aid
- Baxa Repeater Pump
- Sebra Tube Sealer
- 2-8 °C Refrigerator
- -80 °C Freezer
- -20 °C Freezer
- Timer

[00589] Equipment List: CM2 Preparation/Day 11 REP Seed

- Magnehelic Gauge
- Biological Safety Cabinet (BSC)
- Incubator
- Incubator
- CO2 Analyzer

- Dry Bath
- Water Bath
- CytoTherm
- Welder
- Gatherex
- NC200 NucleoCounter
- Baxa Repeater Pump
- Sebra Tube Sealer Balance

[00590] Equipment List: CM2 Preparation/Day 11 REP Seed

- Centrifuge
- Micropipetter (100-1000µL)
- Pipet-Aid
- Timer
- 2-8°C Refrigerator
- -80°C Freezer
- Controlled Rate Freezer
- LN2 Storage Freezer (Quarantine)
- -20°C Freezer

[00591] Equipment List: CM4 Preparation/Day 16

- Magnehelic Gauge
- Biological Safety Cabinet (BSC)
- Incubator
- Incubator
- CO2 Analyzer
- Welder
- Welder
- Gatherex
- NC200 NucleoCounter
- Baxa Repeater Pump
- Sebra Tube Sealer Balance
- Micropipetter (100-1000µL)
- Pipet-Aid
- 2-8°C Refrigerator
- -80°C Freezer

[00592] Equipment List: Day 22 Formulation, Fill, Cryopreservation

- Magnehelic Gauge
- Biological Safety Cabinet (BSC)

- Incubator
- Incubator
- CO2 Analyzer
- Welder
- Gatherex
- NC200 NucleoCounter
- Baxa Repeater Pump
- Sebra Tube Sealer Balance
- Micropipetter (20-200µL) Pipet-Aid

[00593] Equipment List: Day 22 Formulation, Fill, Cryopreservation

- Pipet-Aid
- 2-8°C Refrigerator
- -80°C Freezer
- Controlled Rate Freezer
- LN2 Storage Freezer
- LN2 Storage Freezer (Quarantine)
- LOVO Cell Processing System

7.0 MATERIALS

[00594] Materials: Day 0 CM1 Media Preparation /Tumor Wash Preparation/Tumor

[00595] Dissection

- Disposable Scalpels, Sterile
- 50 mL Serological Pipets, Sterile
- 1 mL Serological Plastic Pipet, Sterile
- 10 mL Serological Pipet, Sterile
- Centrifuge Tube, 50mL, 28x114mm, Conical Base, Screw Cap, PP, Sterile
- 25 mL Serological Pipet, Sterile
- 5 mL Serological Pipet, Sterile
- MF75 Series, Disposable Tissue Culture Filter, 1000 mL, aPES Filter, 0.2 µm, Sterile
- Pipets, Serological 100 mL
- 2-mercaptoethanol 1000X, liquid, 55 mM in D-PBS
- Hank's Balanced Sodium Salt Solution (1X), Liquid, w/o Calcium Chloride, Magnesium Chloride, Magnesium Sulfate
- GlutaMAX 1-200 mM (100X), liquid
- ART Barrier Pipet Tips, 1000µL, Individually Wrapped, Sterile
- 150mm Petri Dish, Extra-Depth, Sterile
- 6-well, Ultra-Low Attachment Plates, 9.5cm^2 Well Growth Area, PS, Sterile
- Thermo Scientific Samco General-Purpose Transfer Pipettes. 7.7mL, Sterile
- Repeater Pump Fluid Transfer Set Male Luer Lock End

- Long Forceps 8", Sterile
- Gentamicin Sulfate, 50mg/mL stock
- Scientific Disposable Forceps, 4.5", Stainless Steel, Sterile
- 100 mm petri dish, Sterile Extra Depth
- Pumpmatic Liquid-Dispensing System
- Gentamicin Sulfate, 50mg/mL stock
- Syringe Cap Dual Function, Red
- RPMI-1640, 1 L Bottle
- G-Rex 100M Flask Closed System
- Sterile rulers
- Reconstituted IL-2
- Human Tumor Sample, Head and Neck □ N/A
- Human Tumor Sample, Cervical □ N/A
- GemCell Human Serum AB, Heat Inactivated □ N/A
- Human Tumor Sample, Melanoma
- GemCell Human Serum AB, Heat Inactivated

[00596] Materials: CM2 Preparation/Day 11 REP Seed

- Luer-Lok Syringe, 60 mL Sterile Needle 16G x 1.5” Sterile
- 50 mL Serological Pipets, Sterile
- 1 mL Serological Plastic, Pipet, Sterile
- Nunc Internally Threaded Cryotube Vials, Sterile
- 10 mL Serological Pipet, Sterile
- Centrifuge Tube, 15 mL
- Centrifuge Tube, 50 mL
- Pipets, Serological 100 mL
- Syringe, 1 cc Sterile Luer-Lok
- 3 mL Syringe, Luer-Lok Tip, Sterile
- 5 mL Serological Pipet, Sterile
- Nalgene *MF75* Series Filter Unit Receiver, 250mL, Sterile
- Nalgene MF75 Series Filter Unit Receiver, 500mL, Sterile
- 1000mL Nalgene Rapid- Flow Sterile Disposable Filter Unit, 0.22 µm PES
- CryoStor CS-10
- 2-mercaptoethanol 1000X Liquid, 55 mM in D-PBS
- GlutaMAX 1-200 mM (100X), liquid
- 1,000 µL ART Barrier Sterile Pipet Tips, Individual Wrap
- VIA1 Cassettes
- Transfer Pack Container, 1000 mL w/ Coupler, Sterile
- Transfer Pack 300 mL w/ Coupler
- Sterile Alcohol Pads
- Repeater Pump Fluid Transfer Set Male Luer Lock Ends
- CTS AIM V 1L Bottle

- MACS GMP CD3 pure (OKT-3)
- Gentamicin Sulfate, 50mg/mL stock
- 4" Tubing w/ Piercing Pin and Syringe Adapter
- Syringe Only Luer-Lok 10 mL
- Tubing, Four Spike Male Luer Manifold
- Gravity Blood Administration Set Y-type with No injection site, 170µm blood filter
- Pumpmatic Liquid-Dispensing System
- 10L Labtainer 3 Port Bag
- Gentamicin Sulfate, 50mg/mL stock
- 100mL Syringe
- 3000mL Culture Bag
- Origen Cell Connect CC2
- Syringe Cap Dual Function Red
- RPMI-1640, 1L Bottle
- G-Rex 500M Flask Closed System
- Reconstituted IL-2
- Allogeneic Irradiated Feeder Cells
- Allogeneic Irradiated Feeder Cells
- Human Serum, type AB(HI) Gemini
- Human Serum, type AB(HI) Gemini

[00597] Materials: CM4 Preparation/Day 16

- Luer-Lok Syringe, 60 mL Sterile
- 1 mL Serological Plastic Pipet, Sterile
- Nunc Internally Threaded Cyrotube Vials, Sterile
- 10 mL Serological Pipets, Sterile
- Centrifuge Tube, 15mL
- Centrifuge Tube, 50 mL
- Pipets, Serological 100 mL
- Syringe with Luer-Lock, sterile, 3mL
- 5 mL Serological Pipet, Sterile
- Syringe only Luer-Lok 10 mL
- Nalgene *MF75* Series
- Filter Unit Receiver, 250mL, Sterile
- GlutaMAX1-200 mM (100X), liquid
- ART Barrier Pipet Tips, 1000 µL, Individually Wrapped, Sterile
- VIA1 Cassettes

[00598] Materials: CM4 Preparation/Day 16

- Transfer Pack Container, 1000 mL with Coupler, Sterile
- Sterile Alcohol Pads

- Repeater Pump Fluid Transfer Set Male Luer Lock Ends
- CTS AIM-V 1000mL N/A
- Plasma Transfer Set 4" Tubing with Female Luer Adapter
- 30mL Luer-Lok Sterile Syringe
- CTS AIM V 10L bag
- Pumpmatic Liquid-Dispensing System
- 10L Labtainer 3 Port Bag
- Syringe Cap Dual Function Red
- G-Rex500M Flask Closed System
- Reconstituted IL-2

[00599] Materials: Day 22 Formulation, Fill, Cryopreservation

- Luer-Lok Syringe, 60 mL Sterile
- Needle 16G x 1.5" Sterile
- 50 mL Serological Pipets, Sterile
- Nunc Internally Threaded Cryotubes Vials, Sterile
- 10 mL Serological Pipet, Sterile
- Centrifuge Tube, 15 mL
- Centrifuge Tube, 50 mL
- Syringe, 1cc Sterile Luer-Lok
- 3 mL Syringe, Luer-Lok Tip, Sterile
- 25 mL Serological Pipet, Sterile
- 5 mL Serological Pipet, Sterile
- Syringe only Luer-Lok 10 mL
- Pipets, Serological 100 mL
- ART Barrier Sterile Pipet Tips, 200 µL Individual Wrap
- VIA1-Cassettes
- Plasma-Lyte A Injection 1L
- LOVO Cell Washing Disposable Set
- LOVO Ancillary Bag Kit
- Sterile Alcohol Pads
- Repeater Pump Fluid Transfer Set Male Luer Lock Ends
- CTS AIM V 1L Bottle
- Human Albumin 25%
- Plasma Transfer Set 4" Tubing with Female Luer Adapter
- Tubing, Four Male Luer Manifold
- Gravity Blood Administration
- Set Y-type with No injection site, 170µm blood filter
- Pumpmatic Liquid-Dispensing System
- 10L Labtainer 3 Port Bag
- 100mL Syringe

- Cryo bag CS750
- 3L Culture Bag
- Origen Cell Connect CC2
- Syringe Cap Dual Function Red
- Cryostor CS10, 100mL Bag
- Dispensing Spike, Vented
- Reconstituted IL-2

[00600] PROCESS

8.1 Day 0 CM1 Media Preparation

8.1.1 Checked room sanitization, line clearance, and materials. Confirmed room sanitization,
8.1.2 Ensured completion of pre-processing table.
8.1.3 Environmental Monitoring. Prior to processing, ensured pre-process environmental monitoring had been initiated.
8.1.4 Prepared RPMI 1640 Media In the BSC, using an appropriately sized pipette, removed 100.0 mL from 1000 mL RPMI 1640 Media and placed into an appropriately sized container labeled "Waste".
8.1.5 In the BSC added reagents to RPMI 1640 Media bottle. Added the following reagents to the RPMI 1640 Media bottle as shown in in table. Recorded volumes added.
Amount Added per bottle: Heat Inactivated Human AB Serum (100.0 mL); GlutaMax (10.0 mL); Gentamicin sulfate, 50 mg/mL (1.0 mL); 2-mercaptoethanol (1.0 mL)
8.1.6 Mixed Media. Capped RPMI 1640 Media bottle from Step 8.1.5 and swirled bottle to ensure reagents were mixed thoroughly.
8.1.7 Filtered RPMI media. Filtered RPMI 1640 Media from Step 8.1.6 through 1L 0.22-micron filter unit.
8.1.8 Labeled filtered media. Aseptically capped the filtered media and labeled with the following information.
8.1.9 Removed unnecessary materials from BSC. Passed out media reagents from BSC, left Gentamicin Sulfate and HBSS in BSC for Formulated Wash Media preparation in Section 8.2.
8.1.10 Stored unused consumables. Transferred any remaining opened/thawed media reagents to appropriate storage conditions or disposed into waste.
NOTE: Assigned the appropriate open expiry to media reagents per Process Note 5.9 and labeled with batch record lot number
8.1.11 Thawed IL-2 aliquot. Thawed one 1.1 mL IL-2 aliquot ($6x10^6$ IU/mL) (BR71424) until all ice had melted. Recorded IL-2: Lot # and Expiry (NOTE: Ensured IL-2 label was attached).
8.1.12 Transferred IL-2 stock solution to media. In the BSC, transferred 1.0 mL of IL-2 stock solution to the CM1 Day 0 Media Bottle prepared in Step 8.1.8. Added CM1 Day 0 Media 1 bottle and IL-2 ($6x10^6$ IU/mL) 1.0 mL.
8.1.13 Mixed and Relabeled. Capped and swirled the bottle to mix media containing IL-2. Relabeled as "Complete CM1 Day 0 Media" and assigned new lot number.

8.1.14 Sample Media per Sample Plan. Removed 20.0 mL of media using an appropriately sized pipette and dispensed into a 50mL conical tube.
8.1.15 Labeled and stored. Sample labeled with sample plan inventory label and stored "Media Retain" sample at 2-8ºC until submitted to Login for testing per Sample Plan.
8.1.16 Signed for Sampling. Ensured that LIMS sample plan sheet was completed for removal of the sample.
8.1.17 Prepared "Tissue Pieces" conical tube. In BSC, transferred 25.0 mL of "Complete CM1 Day 0 Media" (prepared in Step 8.1.13) to a 50 mL conical tube. Labeled the tube as "Tissue Pieces" and batch record lot number.
8.1.18 Passed G-Rex100MCS into BSC. Aseptically passed G-Rex100MCS (W3013130) into the BSC.
8.1.19 Prepared G-Rex100MCS. In the BSC, closed all clamps on the G-Rex100MCS, leaving vent filter clamp open.
8.1.20 Prepared G-Rex100MCS. Connected the red line of G-Rex100MCS flask to the larger diameter end of the repeater pump fluid transfer set (W3009497) via luer connection.
8.1.21 Prepared Baxa Pump. Staged Baxa pump next to BSC. Removed pump tubing section of repeater pump fluid transfer set from BSC and installed in repeater pump.
8.1.22 Prepared to pump media. Within the BSC, removed the syringe from Pumpmatic Liquid-Dispensing System (PLDS) (W3012720) and discarded.
NOTE: Ensured to not compromise the sterility of the PLDS pipette.
8.1.23 Prepared to pump media. Connected PLDS pipette to the smaller diameter end of repeater pump fluid transfer set via luer connection and placed pipette tip in "Complete CM1 Day 0 Media" (prepared in Step 8.1.13) for aspiration.
Opened all clamps between media and G-Rex100MCS.
8.1.24 Pumped Complete CM1 media into G-Rex100MCS flask. Set the pump speed to "High" and "9" and pumped all Complete CM1 Day 0 Media into G-Rex100MCS flask. Once all media was transferred, cleared the line and stopped pump.
8.1.25 Disconnected pump from flask. Ensured all clamps were closed on the flask, except vent filter. Removed the repeater pump fluid transfer set from the red media line, and placed a red cap (W3012845) on the red media line.
8.1.26 Heated seal. Removed G-Rex100MCS flask from BSC, heated seal (per Process Note 5.12) off the red cap from the red line near the terminal luer.
8.1.27 Labeled G-Rex100MCS. Labeled G-Rex100MCS flask with QA provided in-process "Day 0" label. Attached sample "Day 0" label below.
8.1.28 Monitored Incubator. Incubator parameters: Temperature LED Display: 37.0±2.0 ºC; CO2 Percentage: 5.0±1.5 %CO2
8.1.29 Warmed Media. Placed the 50mL conical tube labeled "Tissue Fragments" prepared in Step 8.1.17 and the G-Rex100MCS prepared in Step 8.1.27 in incubator for ≥ 30 minutes of warming.
Recorded warming times below. Recorded if Warm Time was ≥ 30 minutes (Yes/No).
[Tissue Fragments Conical or GRex100MCS]
8.1.30 Reviewed Section 8.1.

8.2 Day 0 Tumor Wash Media Preparation

8.2.1 Added Gentamicin to HBSS. In the BSC, added 5.0 mL Gentamicin (W3009832 or W3012735) to 1 x 500 mL HBSS Media (W3013128) bottle. Recorded volumes. Added per bottle: HBSS (500.0 mL); Gentamicin sulfate, 50
mg/ml (5.0 mL)
8.2.2 Capped HBSS bottle and swirled. Capped HBSS containing gentamicin prepared in Step 8.2.1 and swirled bottle to ensure reagents are mixed thoroughly.
8.2.3 Filtered Solution. Filtered HBSS containing gentamicin prepared in Step 8.2.1 through a 1L 0.22-micron filter unit (W1218810).
8.2.4 Aseptically capped the filtered media and label. Aseptically capped the filtered media and labeled with the following information. Proceeded to SECTION 8.3.
8.2.5 Reviewed Section 8.2.

8.3 Day 0 Tumor Processing

8.3.1 Obtained Tumor. Obtained tumor specimen from QAR and transferred into suite at 2-8°C immediately for processing. Ensured all necessary information is recorded on the Tumor Shipping Batch Record.
8.3.2 Recorded Tumor Information.
8.3.3 Affixed Tumor Label. Affixed tumor Attachment. QAR release sticker below. Attached Tumor Shipping Batch Record as #5.
8.3.4 Passed in necessary materials for tumor dissection into the BSC.
8.3.5 Opened Materials. Opened all materials inside the BSC, ensuring not to compromise the sterility of the items.
8.3.6 Labeled Materials. Labeled three 50ml conical tubes: the first as "Forceps," the second as "Scalpel," and the third as "Fresh Tumor Wash Media". Labeled 5 x 100 mm petri dishes as "Wash 1," "Wash 2," "Wash 3," "Holding," and "Unfavorable." Labeled one 6 well plate as "Favorable Intermediate Fragments."
8.3.7 Aliquoted Tumor Wash Media. Using an appropriately sized pipette, transferred 5.0 mL of "Tumor Wash Media" prepared in Step 8.2.4 into each well of one 6-well plate for favorable intermediate tumor fragments (30.0 mL total). NOTE: The forceps and scalpels were stored in their respective tumor wash media conicals as needed during the tumor washing and dissection processes.
8.3.8 Aliquoted Tumor Wash Media. Using an appropriately sized pipette, transferred 50.0 mL of "Tumor Wash Media" prepared in Step 8.2.4 into each 100 mm petri dish for "Wash 1," "Wash 2," "Wash 3," and "Holding" (200.0 mL total).
8.3.9 Aliquoted Tumor Wash Media. Using an appropriately sized pipette, transfer 20.0 mL of "Tumor Wash Media" prepared in Step 8.2.4 into each 50 mL conical (60.0 mL total).
8.3.10 Prepared Lids for Tumor Pieces. Aseptically removed lids from two 6-well plates. The lids were utilized for selected tumor pieces. NOTE: Throughout tumor processing, DID NOT cross over open tissue culture plates and lids.
8.3.11 Passed the tumor into the BSC. Aseptically passed the tumor into the BSC. Recorded processing start time.

8.3.12 Tumor Wash 1 Using 8" forceps (W3009771), removed the tumor from the specimen bottle and transferred to the "Wash 1" dish prepared in Step 8.3.8.
NOTE: Retained the solution in specimen bottle.
8.3.13 Tumor Wash 1 Using forceps, gently washed tumor time from timer below: specimen and allowed it to sit for ≥ 3 minutes. Recorded wash time (MM:SS).
8.3.14 Prepared Bioburden Sample per Sample Plan. Transferred 20.0 mL (or available volume) of solution from the tumor specimen bottle into a 50mL conical per sample plan.
8.3.15 Labeled and stored sample. Labeled with sample plan inventory label and stored bioburden sample collected in Step 8.3.14 at 2-8ºC until submitted for testing.
8.3.16 Signed for sampling. Ensured that LIMS sample plan sheet was completed for removal of the sample.
8.3.17 Tumor Wash 2. Using a new set of forceps removed the tumor from the "Wash 1" dish and transferred to the "Wash 2" dish prepared in Step 8.3.8.
8.3.18 Tumor Wash 2. Using forceps, washed tumor specimen by gently agitating for ≥ 3 minutes and allowed it to sit. Recorded time.
8.3.19 Prepared drops of Tumor Wash Media for desired tumor pieces. Using a transfer pipette, placed 4 individual drops of Tumor Wash Media from the conical prepared in Step 8.3.9 into each of the 6 circles on the upturned lids of the 6-well plates (2 lids). Placed an extra drop on two circles for a total of 50 drops.
8.3.20 Tumor Wash 3. Using forceps, removed the tumor from the "Wash 2" dish and transferred to the "Wash 3" dish prepared in Step 8.3.8.
8.3.21 Tumor Wash 3. Using forceps, washed tumor specimen by gently agitating and allowed it to sit for ≥ 3 minutes. Recorded time.
8.3.22 Prepared tumor dissection dish. Placed a ruler under 150 mm dish lid.
8.3.23 Transferred Tumor to Dissection Dish. Using forceps, aseptically transferred tumor specimen to the 150 mm dissection dish lid.
8.3.24 Measured Tumor. Arranged all pieces of tumor specimen end to end and recorded the approximate overall length and number of fragments. Took a clear picture of each tumor specimen.
8.3.25 Assessed Tumor. Assessed the tumor for necrotic/fatty tissue. Assessed whether > 30% of entire tumor area observed to be necrotic and/or fatty tissue; if yes, contacted area management to ensure tumor was of appropriate size, then proceeded to Step 8.3.26. Assessed whether < 30% of entire tumor area were observed to be necrotic or fatty tissue; if yes, proceeded to Step 8.3.27 and clean-up dissection was NOT performed.
8.3.26 If applicable: Clean-Up Dissection. If tumor was large and >30% of tissue exterior was observed to be necrotic/fatty, performed "clean up dissection" by removing necrotic/fatty tissue while preserving tumor inner structure using a combination of scalpel and/or forceps. NOTE: To maintain tumor internal structure, used only vertical cutting pressure. Did not cut in a sawing motion with scalpel. NOTE: Fat, necrotic, and extraneous tissue were placed in unfavorable dish.
8.3.27 Dissect TumorUsing a combination of scalpel and/or forceps, cut the tumor specimen into even, appropriately sized fragments (up to 6 intermediate fragments). NOTE: To maintain tumor internal structure, use only vertical

cutting pressure. Did not cut in a sawing motion with scalpel. NOTE: Ensured to keep non-dissected intermediate fragments completely submerged in "Tumor Wash Media" (prepared in Step 8.2.4).
8.3.28 Transferred intermediate tumor fragments. Transferred each intermediate fragment to the "holding" dish from Step 8.3.8.
8.3.29 Dissected Tumor Fragments. Manipulated one intermediate fragment at a time, dissected the tumor intermediate fragment in the dissection dish into pieces approximately 3x3x3mm in size, minimizing the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. NOTE: To maintain tumor internal structure, used only vertical cutting pressure. Did not cut in a sawing motion with scalpel.
8.3.30 Selected Tumor Pieces. Selected up to eight (8) tumor pieces without hemorrhagic, necrotic, and/or fatty tissue. Used the ruler for reference. Continued dissection until 8 favorable pieces have been obtained, or the entire intermediate fragment has been dissected. Transferred each selected piece to one of the drops of "Tumor Wash Media" prepared in Step 8.3.19.
8.3.31 Stored Intermediate Fragments to Prevent Drying. After selecting up to eight (8) pieces from the intermediate fragment, placed remnants of intermediate fragment into a new single well of "Favorable Intermediate Fragments" 6-well plate prepared in Step 8.3.7. NOTE: Fatty or necrotic tissue was placed in the "Unfavorable" dish (prepared in step 8.3.6).
8.3.32 Repeated Intermediate Fragment Dissection. Proceeded to the next intermediate fragment, repeated Steps 8.3.29-8.3.31 until all intermediate fragments had been processed, obtained fresh scalpels and forceps as needed.

8.3.33 Determined number of pieces collected. If desirable tissue remains, selected additional Favorable Tumor Pieces from the "favorable intermediate fragments" 6-well plate to fill the drops for a maximum of 50 pieces. Recorded the total number of dissected pieces created. NOTE: Ensuring to keep the tumor intermediate fragments hydrated with Wash Medium as necessary throughout dissection. Recorded Total quantity of dissected pieces collected.
8.3.34 Removed Conical Tube from Incubator. Removed the "Tissue Pieces" 50mL conical tube from the incubator. Recorded time in Step 8.1.29. Ensured conical tube was warmed for $\geq$30 min.
8.3.35 Prepared Conical Tube. Passed "Tissue Pieces" 50mL conical into the BSC, ensuring not to compromise the sterility of open processing surfaces.
8.3.36 Transferred Tumor Pieces to 50mL Conical Tube. Using a transfer pipette, scalpel, forceps or combination, transferred the selected 50 best tumor fragments from favorable dish lids to the "Tissue Pieces" 50 mL conical tube. NOTE: If a tumor piece was dropped during transfer and desirable tissue remains, additional pieces from the favorable tumor intermediate fragment wells were added. Recorded numbers of pieces.
8.3.37 Prepared BSC for G- REX100MCS. Removed all unnecessary items from BSC for vessel seed, retaining the favorable tissue plates if they contained extra fragments.
8.3.38 Removed G-REX100MCS from Incubator. Removed G-Rex100MCS containing media from incubator. Completed Step 8.1.29.
8.3.39 Passed flask into BSC. Aseptically passed G-Rex100MCS flask into the BSC. NOTE: When transferring the flask, did not hold from the lid or the

bottom of the vessel. Transferred the vessel by handling the sides. NOTE: Only utilized IPA WIPES when handling G-Rex flasks.
8.3.40 Added tumor fragments to G-Rex100MCS flask. In the BSC, lifted G-Rex100MCS flask cap, ensuring that sterility of internal tubing was maintained.
Swirled conical tube with tumor pieces to suspend and quickly poured the contents into the G-Rex100MCS flask.
8.3.41 Evenly distributed pieces. Ensured that the tumor pieces were evenly distributed across the membrane of the flask. Gently tilted the flask back and forth if necessary to evenly distribute the tumor pieces.
8.3.42 Recorded total number of tumor fragments in vessel. Recorded number of tumor fragments on bottom membrane of vessel and number of observed to be floating in vessel. NOTE: If the number of fragments seeded were NOT equivalent to number of collected in Step 8.3.36H, contacted Area Management, and document in Section 10.0.
8.3.43 Incubate G-Rex flask Incubated G-Rex100MCS at the following parameters: Incubated G-Rex flask: Temperature LED Display: 37.0±2.0 ºC; CO2 Percentage: 5.0±1.5 %CO2
8.3.44 Calculated incubation window. Performed calculations to determine the proper time to remove G-Rex100MCS incubator on Day 11. Calculations: Time of incubation; lower limit = time of incubation + 252 hours; upper limit = time of incubation + 276 hours
8.3.45 Environmental Monitoring. After processing, verified BSC and personnel monitoring were performed.
8.3.46 Discarded materials. Stored remaining unwarmed media at 2-8°C and labeled. After process was complete, discarded any remaining warmed media and thawed aliquots of IL-2.
8.3.47 Sample submission. Ensured all Day 0 samples were submitted to Login and transferred in LIMS.
8.3.48 Review Section 8.3.

8.4 Day 11 – Media Preparation
8.4.1 Checked room, sanitization, line clearance, and materials. Confirmed room sanitization, line clearance, and that materials are within expiry.
8.4.2 Pre-processing table. Equipment list: BSC; Balance; Sebra Tube Sealer; Gatherex™ Media Removal and Cell Recovery Device; Ensure QA provided placard is placed on the appropriate BSC; Ensure QA provided placard lot number and patient ID display matches the lot number and patient ID in this Batch Record.
8.4.3 Monitored Incubator. Monitored Incubator. Incubator parameters: Temperature LED Display: 37.0±2.0 ºC; CO2 Percentage: 5.0±1.5 %CO2. NOTE: Section 8.4 may be run concurrently with section 8.5.
8.4.4 Warmed media. Warmed 3x 1000 mL RPMI 1640 Media (W3013112) bottles and 3x 1000 mL AIM-V (W3009501) bottles in an incubator for ≥ 30 minutes. Recorded time. Media: RPMI 1640 and AIM-V. NOTE: Placed an additional 1x1000 ml bottle of AIM-V Media (W3009501) at room temperature for use in Step 8.5.34. Labeled the bottle “For Cell Count Dilutions Only” and the batch record lot number.
8.4.5 Environmental monitoring. Prior to processing, ensured pre-process environmental monitoring was performed as per SOP-00344.

8.4.6 Removed RPMI 1640 Media from incubator. Removed the RPMI 1640 Media when time was reached. Record end incubation time in Step 8.4.4. Ensure media was warmed for ≥30 min.
8.4.7 Prepared RPMI 1640 Media. In the BSC, removed 100.0 mL from each of the three pre-warmed 1000 mL RPMI 1640 Media bottles and placed into an appropriately sized container labeled "Waste".
8.4.8 In BSC add reagents to RPMI 1640 Media bottle. In the BSC added the following reagents to each of the three RPMI 1640 Media bottles. Recorded volumes added to each bottle. GemCell Human serum, Heat Inactivated Type AB (100.0 mL), GlutaMax (10.0 mL), Gentamicin sulfate, 50 mg/ml (1.0 mL), 2-mercaptoethanol (1.0 mL)
8.4.9 Filter Media. Caped bottles from Step 8.4.8 and swirled to ensure reagents were mixed thoroughly. Filtered each bottle of media through a separate 1L 0.22-micron filter unit.
8.4.10 Labeled filtered media. Aseptically capped the filtered media and labeled each bottle with CM1 Day 11 Media.
8.4.11 Thawed IL-2 aliquot. Thawed 3 x 1.1mL aliquots of IL-2 (6x106 IU/mL) (BR71424) until all ice had melted Recorded IL 2 lot # and Expiry. NOTE: EnsureIL-2 label is attached.
8.4.12 Removed AIM-V Media from the incubator. Removed the three bottles of AIM-V Media from the incubator. Recorded end incubation time in Step 8.4.4. Ensured media had been warmed for ≥ 30 minutes.
8.4.13 Add IL-2 to AIM-V. In the BSC, using a micropipette, added 3.0mL of thawed IL-2 into one 1L bottle of pre-warmed AIM-V media. Rinse micropipette tip with media after dispensing IL-2. Use a new sterile micropipette tip for each aliquot. Recorded the total volume added. Labeled bottle as "AIM-V Containing IL-2".
8.4.14 Transferred materials. Aseptically transferred a 10L Labtainer Bag and a repeater pump transfer set into the BSC.
8.4.15 Prepared 10L Labtainer media bag. Closed all lines on a 10L Labtainer bag. Attached the larger diameter tubing end of a repeater pump transfer set to the middle female port of the 10L Labtainer Bag via luer lock connection.
8.4.16 Prepare Baxa pump. Staged the Baxa pump next to the BSC. Fed the transfer set tubing through the Baxa pump situated outside of the BSC. Set the Baxa Pump to "High" and "9".
8.4.17 Prepared 10L Labtainer media bag. In BSC, removed syringe from Pumpmatic Liquid-Dispensing System (PLDS) and discarded. NOTE: Ensured to not compromise the sterility of the PLDS pipette.
8.4.18 Prepared 10L Labtainer media bag. Connected PLDS pipette to smaller diameter end of repeater pump fluid transfer set via luer connection and placed pipette tip in AIM-V media containing IL-2 bottle (prepared in Step 8.4.13) for aspiration. Opened all clamps between media bottle and 10L Labtainer.
8.4.19 Pumped media into 10L Labtainer. In the BSC, using the PLDS, transfer pre-warmed AIM-V media containing IL-2 prepared in Step 8.4.13, as well as two additional AIM-V bottles into the 10L Labtainer bag. Added the three bottles of filtered CM1 Day 11 Media from Step 8.4.10. After addition of final bottle, cleared the line to the bag. NOTE: Stopped the pump between addition of each bottle of media.

8.4.20 Removed pumpmatic from Labtainer bag. Removed PLDS from the transfer set and placed a red cap on the luer of the line in the BSC.
8.4.21 Mixed media. Gently massaged the bag to mix.
8.4.22 Labeled media. In the BSC, labeled the media bag with the following information. Expiration date was 24 hours from the preparation date.
8.4.23 Sample media per sample plan. In the BSC, attached a 60mL syringe to the available female port of the "Complete CM2 Day 11 Media" bag prepared in step 8.4.22. Removed 20.0mL of media and place in a 50mL conical tube. Placed a red cap on the female port of the "Complete CM2 Day 11 Media" Bag.
8.4.24 Labeled and stored sample. Labeled with sample plan inventory label and stored Media Retain Sample at 2-8ºC until submitted to Login for testing.
8.4.25 Sign for Sampling. Ensured that LIMS sample plan sheet was completed for removal of the sample.
8.4.26 Sealed the transfer set line. Outside the BSC, heat sealed off (per Process Note 5.12) the red cap on the transfer set line, close to red cap. Kept the transfer set on the bag.
8.4.27 Prepared Cell Count Dilution Tubes In the BSC, added 4.5mL of AIM-V Media that had been labelled with "For Cell Count Dilutions" and lot number to four 15mL conical tubes. Labeled the tubes with the lot number and tube number (1-4). Labeled 4 cryovials "Feeder" and vial number (1-4). Kept vials under BSC to be used in Step 8.5.30.
8.4.28 Transferred reagents from the BSC to 2-8°C. Transferred any remaining 2-mercaptoethanol, GlutaMax, and human serum from the BSC to 2-8°C. Ensured all reagents were labeled with the batch record lot number, and the appropriate open expiry per Process Note 5.9.
8.4.29 Prepared 1L Transfer Pack. Outside of the BSC weld (per Process Note 5.11) a 1L Transfer Pack to the transfer set attached to the "Complete CM2 Day 11 Media" bag prepared in step 8.4.22. Labeled transfer pack as "Feeder Cell CM2 Media" and lot number.
8.4.30 Prepared 1L Transfer Pack. Made a mark on the tubing of the 1L Transfer Pack tubing a few inches away from the bag. Placed the empty Transfer Pack onto the scale so that the tubing was on the scale to the point of the mark.
8.4.31 Tared scale. Tared the scale and left the empty Transfer Pack on the scale.
8.4.32 Prepared feeder cell transfer pack. Set the Baxa pump to "Medium" and "4." Pumped 500.0 ±5.0mL of "Complete CM2 Day 11" media prepared in Step 8.4.22 into Cell CM2 Media" transfer pack. Measured by weight and recorded the volume of Complete CM2 media added to the Transfer Pack.
8.4.33 Heated seal line. Once filled, heated seal the line per Process Note 5.12. Separated CM2 Day 11 media bag with transfer set from feeder cell media transfer pack, kept weld toward 1L transfer pack.
8.4.34 If applicable: Incubated feeder cell media transfer pack. When applicable, placed the "Feeder Cell CM2 Media" transfer pack in incubator until used in Step 8.6.6.
8.4.35 Incubated Complete CM2 Day 11 Media. Placed "Complete CM2 Day 11 Media" prepared in Step 8.4.22 in incubator untiluse in Step 8.7.2.
8.4.36 Reviewed Section 8.4.

8.5 Day 11 - TIL Harvest

8.5.1 Preprocessing table. Monitored incubator. Incubator parameters: Temperature LED Display: 37.0±2.0 ℃; CO2 Percentage: 5.0±1.5 %CO2. NOTE: Section 8.5 may be run concurrently with Sections 8.4 and 8.6.
8.5.2 Removed G-Rex100MCS from incubator. Performed check below to ensure incubation parameters are met before removing G-Rex100MCS from incubator. Lower limit from Step 8.3.44 B. Upper limit from Step 8.3.44 C. Record Time of Removal from incubator. Determined: Is 8.3.44 B ≤ Time of Removal from incubator < Step 8.3.44 C? *IF NO CONTACT AREA MANAGEMENT. Carefully removed G-Rex100MCS from incubator and ensured all clamps were closed except large filter line. Recorded processing start time.
8.5.3 Prepared 300mL Transfer Pack. Labeled a 300mL Transfer pack as "TIL Suspension".
8.5.4 Prepared 300mL Transfer Pack. Sterile welded (per Process Note 5.11) the TIL Suspension transfer (single line) of a Gravity Blood Filter. See, for example.
8.5.5 Prepared 300mL Transfer Pack. Placed the 300mL Transfer Pack on a scale and record dry weight.
8.5.6 Prepared 1L Transfer Pack. Labeled 1L Transfer Pack as "Supernatant" and Lot number.
8.5.7 Welded transfer packs to G-Rex100MCS. Sterile welded (per Process Note 5.11) the red media removal line from the G-Rex100MCS to the "Supernatant" transfer pack. Sterile welded the clear cell removal line from the G-Rex100MCS to one of the two spike lines on the top of the blood filter connected to the "TIL Suspension" transfer pack prepared in Step 8.5.4. See, for example.
8.5.8 GatheRex Setup. Placed G-Rex100MCS on the left side of the GatheRex and the "Supernatant" and "TIL Suspension" transfer packs to the right side.
8.5.9 GatheRex Setup. Install the red media removal line from the G Rex100MCS to the top clamp (marked with a red line) and tubing guides on the GatheRex. Installed the clear harvest line from the G-Rex100MCS to the bottom clamp (marked with a blue line) and tubing guides on the GatheRex.
8.5.10 GatheRex Setup. Attached the gas line from the GatheRex to the sterile filter of the G-Rex100MCS flask. NOTE: Before removing the supernatant from the G-Rex100MCS flask, ensured all clamps on the cell removal lines were closed.
8.5.11 Volume Reduction of G-Rex100MCS. Transferred ~900 mL of culture supernatant from the G-Rex100MCS to the 1L Transfer Pack. Visually inspect G-Rex100MCS flask to ensure flask is level and media has been reduced to the end of the aspirating dip tube. NOTE: If the Gatherex stops prematurely, it was restarted by pressing the button with the arrow pointing to the right again.
8.5.12 Prepare flask for TIL Harvest. After removal of the supernatant, closed all clamps to the red line.
8.5.13 Initiation of TIL Harvest. Recorded the start time of the TIL harvest.
8.5.14 Initiation of TIL Harvest. Vigorously tapped flask and swirled media to release cells. Performed an inspection of the flask to ensure all cells have detached. NOTE: Contacted area management if cells did not detach.

8.5.15 Initiation of TIL Harvest. Tilt flask away from collection tubing and allowed tumor pieces to settle along edge. Slowly tipped flask toward collection tubing so pieces remained on the opposite side of the flask. NOTE: If the cell collection straw is not at the junction of the wall and bottom membrane, rapping the flask while tilted at a 450 angle is usually sufficient to properly position the straw.
8.5.16 TIL Harvested. Released all clamps leading to the TIL Suspension transfer pack.
8.5.17 TIL Harvested. Using the GatheRex, transferred the cell suspension through the blood filter into the 300mL transfer pack. NOTE: Be sure to maintain the tilted edge until all cells and media are collected.
8.5.18 TIL Harvested. Inspect membrane for adherent cells.
8.5.19 Rinsed flask membrane. Rinsed the bottom of the G-Rex100MCS. Cover ~1/4 of gas exchange membrane with rinse media. NOTE: If tumor pieces obstruct the harvest line, pause collection by pressing the "X" on the cell collection line. Press the "Release Clamps" button on the Gatherex and pick the transfer pack up and gently squeeze with increasing pressure until the fragment is removed. Do not squeeze the bag too hard, as this may cause the line or bag to rupture. Resume collection once obstruction has been removed.
8.5.20 Closed clamps on G-Rex100MCS. Ensured all clamps are closed.
8.5.21 Heat sealed. Heat sealed (per Process Note 5.12) the TIL suspension transfer pack as close to the weld as possible so that the overall tubing length remains approximately the same.
8.5.22 Heat sealed. Heat sealed the "Supernatant" transfer pack per Process Note 5.12. Maintained enough line to weld.
8.5.23 Calculated volume of TIL suspension. Recorded weight of TIL Suspension transfer pack and calculated the volume of cell suspension.
8.5.24 Prepared Supernatant Transfer Pack for Sampling. Welded (per Process Note 5.11) a 4" plasma transfer set to "supernatant" transfer pack, retaining the luer connection on the 4" plasma transfer set, and transferred into the BSC.
8.5.25 Prepared TIL Suspension Transfer Pack for Sampling. Welded (per Process Note 5.11) a 4" plasma transfer set to 300mL "TIL Suspension" transfer pack, retained the luer connection on the 4" plasma transfer set, and transferred into the BSC.
8.5.26 Pulled Bac-T Sample. In the BSC, using an appropriately sized syringe, draw up approximately 20.0 mL of supernatant from the 1L "Supernatant" transfer pack and dispense into a sterile 50mL conical tube labeled "Bac-T." Keep in BSC for use in Step 8.5.27.
8.5.27 Inoculated BacT per Sample Plan. Removed a 1.0 mL sample from the 50mL conical labeled BacT prepared in Step 8.5.26 using an appropriately sized syringe and inoculated the anaerobic bottle. Recorded the time the bottle was inoculated using the space provided on the bottle label. Repeated the above for the aerobic bottle. NOTE: This step may be performed out of sequence.
8.5.28 Labeled and stored sample. Labeled with sample plan inventory label and stored Bac-T sample at room temperature, protected from light, until submitted to Login for testing per Sample Plan. NOTE: Did not cover barcode on bottle with label.

8.5.29 Signed for Sampling. Ensured that LIMS sample plan sheet was completed for removal of the sample.
8.5.30 TIL Cell Count Samples. Labeled 4 cryovials with vial number (1-4). Using separate 3mL syringes, pulled 4x1.0mL cell count samples from TIL Suspension Transfer Pack using the luer connection, and placed in respective cryovials.
8.5.31 Closed the luer connection. Placed a red cap (W3012845) on the line.
8.5.32 Incubated TIL. Placed TIL Transfer Pack in incubator until needed.
8.5.33 Perform Cell Counts Perform cell counts and calculations utilizing NC-200 and Process Note 5.14. Perform initial cell counts undiluted.
8.5.34 Recorded Cell Count sample volumes. NOTE: If no dilution needed, "Sample [µL]" = 200, "Dilution [µL]" = 0.
8.5.35 Determined Multiplication Factor. Total cell count sample Volume: 8.5.34A + 8.5.34B. Multiplication Factor C ÷ 8.5.34A.
8.5.36 Selected protocols and entered multiplication factors. Ensured the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0
8.5.37 Recorded File Name, Viability and Cell Counts from Nucleoview
8.5.38 Determined the Average of Viable Cell Concentration and Viability of the cell counts performed. Viability (8.5.37A + 8.5.37B) ÷ 2. Viable Cell Concentration (8.5.37C + 8.5.37D) ÷ 2
8.5.39 Determined Upper and Lower Limit for counts. Lower Limit: 8.5.38F x 0.9. Upper Limit: 8.5.38F x 1.1.
8.5.40 Were both counts within acceptable limits? Lower Limit: 8.5.37 C and D ≥ 8.5.39G. Upper Limit: 8.5.37 C and D ≤ 8.5.39H. *If either result was "No" performed second set of counts in steps 8.5.41 – 8.5.48*.
8.5.41 If Applicable: Performed cell counts. Performed cell counts and calculations in utilizing NC-200 and Process Note 5.14. NOTE: Dilution was adjusted according based off the expected concentration of cells. Performed
8.5.42 If Applicable: Recorded Cell Count sample volumes.
8.5.43 If Applicable: Determined Multiplication Factor. Total cell count sample Volume: 8.5.42A + 8.5.42B. Multiplication Factor C ÷ 8.5.42A D
8.5.44 If Applicable: Selected protocols and entered multiplication factors. Ensured the "Viable Cell Count Assay" protocol was selected, all multiplication factors, and sample and diluent volumes were entered. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0.
8.5.45 If Applicable: Recorded Cell Counts from Nucleoview
8.5.46 If Applicable: Determined the Average of Viable Cell Concentration and Viability of the cell counts performed. Determined averaged viable cell concentration.
8.5.47 If Applicable: Determined Upper and Lower Limit for counts. Lower Limit: 8.5.46F x 0.9. Upper Limit: 8.5.46F x 1.1.
8.5.48 If Applicable: Were counts within acceptable limits? Lower Limit: 8.5.45 C and D ≥ 8.5.47G. Upper Limit: 8.5.45 C and D ≤ 8.5.47H. NOTE: If either result is "No" continue to Step 8.5.49 to determine an average.
8.5.49 If Applicable: Determined an average Viable Cell Concentration from all four counts performed. Average Viable Cell Concentration (A+B+C+D) ÷ 4 = AVERAGE

8.5.50 Adjusted Volume of TIL Suspension Calculate the adjusted volume of TIL suspension after removal of cell count samples. Total TIL Cell Volume from Step 8.5.23C (A). Volume of Cell Count Sample Removed (4.0 ml) (B) Adjusted Total TIL Cell Volume C=A-B.

8.5.51 Calculated Total Viable TIL Cells. Average Viable Cell Concentration*: 8.5.38 F* or 8.5.46 F* or *8.5.49E*; Total Volume: 8.5.50; Total Viable Cells: C = A x B. *Circle step reference used to determine Viable Cell Concentration. NOTE: If Total Viable TIL Cells is < $5x10^6$ cells contact Area Management and proceed to Step 8.7.1. If Total Viable TIL Cells is > $5x10^6$, proceed to Step 8.5.52.

8.5.52 Calculation for flow cytometry. If the Total Viable TIL Cell count from Step 8.5.51C was ≥ $4.0x10^7$, calculated the volume to obtain $1.0x10^7$ cells for the flow cytometry sample. *If there are <$4.0x10^7$ cells, N/A the remaining fields in the table. Proceed to Step 8.7.1. Total viable cells required for flow cytometry: $1.0x10^7$ cells. Volume of cells required for flow cytometry: Viable cell concentration from 8.5.51 divided by $1.0x10^7$ cells A.

8.5.53 If Applicable: Removed TIL from incubator . Removed TIL Suspension from incubator and recorded end incubation time in Step 8.5.32.

8.5.54 If Applicable: Removed flow cytometry sample as per Sample Plan. Using an appropriately sized syringe, removed the calculated volume (8.5.52 C) for the phenotyping sample from the TIL Suspension transfer pack and place in a 50mL conical tube.

8.5.55 If Applicable: Labeled and stored flow cytometry sample. Labeled with sample plan inventory label and store Flow Cytometry sample at 2-8°C until submitted to Login for testing per Sampling Plan.

8.5.56 Signed for Sampling. Ensure that LIMS sample plan sheet was completed for removal of the sample.

8.5.57 If Applicable: Recalculated Total Viable Cells and Volume flow. Calculated the remaining Total Viable Cells and remaining volume after the removal of cytometry sample below.

Parameter	Formula	Result
Total Viable TIL	Step 8.5.51C	A. cells
TIL removed for Flow Cytometry	$1x10^7$ cells	B. $1x10^7$ cells
Remaining Total Viable TIL	C = A - B	C. cells
Volume of TIL	Step 8.5.50C	D. mL
Volume of TIL removed	Step 8.5.52 C	E. mL
Remaining Volume of TIL	F = D - E	F. mL

8.5.58 If Applicable: Calculated TIL volume. Calculated the volume of TIL suspension equal to $2.0x10^8$ viable cells.

Total Viable Cells Required	Viable Cell Concentration from Step 8.5.51A	Volume of TIL Suspension containing $2.0x10^8$ viable cells C=A÷B
A. $2.0x10^8$ cells	B. cells/mL	C. mL

8.5.59 If Applicable: Calculated TIL volume to remove Calculate the excess volume of TIL cells to remove.

Total Volume of TIL Suspension from Step 8.5.57F	Volume of suspension containing $2.0x10^8$ TIL from Step 8.5.58C	Volume of excess TIL to Remove C=A-B
A. mL	B. mL	C. mL

8.5.60 If Applicable: Removed excess TIL. In the BSC, using an appropriately sized syringe, remove the calculated volume (Step 8.5.59C) from the TIL Suspension transfer pack. NOTE: Do not use a syringe more than once. Use multiple syringes if applicable. Placed in appropriately sized sterile container and label as date, and lot number. Placed a red cap on the "TIL Suspension" transfer pack line.
8.5.61 If Applicable: Placed TIL in Incubator. Placed TIL Suspension Transfer Pack in incubator until needed. Recorded time.
8.5.62 If Applicable: Calculations. Calculated total excess TIL removed. Step 8.5.51A Volume of TIL to Remove from Step 8.5.59C. Calculated Total Excess TIL removed.

Viable Cell Concentration from Step 8.5.51A	Volume of TIL to Remove from Step 8.5.59C	Total Excess TIL removed C = A x B
A. cells/mL	B. mL	C. cells

8.5.63 If Applicable: Calculations. Calculated amount of CS-10 media to add to excess TIL cells from Step 8.5.62C. Target cell concentration for freezing is 1.0 x 10^8 cells/ml.

Total Excess TIL Removed Step 8.5.62C	Target Concentration to Freeze	Volume of CS-10 to Add (mL) C=A÷B
A. cells	B. $1.0x10^8$ cells/mL	C. mL

8.5.64 If Applicable: Centrifuged excess TIL. Centrifuged the excess TIL cell suspension. Speed: 350 x g. Time: 10:00 minutes. Temperature: Ambient Brake: Full (9). Acceleration: Full (9).
8.5.65 If Applicable: Observed conical tube. Recorded observations: Pellet observed? Supernatant was clear? *NOTE: If either answer was no, contact Area Management.
8.5.66 If Applicable: Added CS-10. In BSC, aseptically aspirate supernatant. Gently tap bottom of tube to resuspend cells in remaining fluid.
8.5.67 If Applicable: Added CS10. Slowly add the volume of CS10 calculated in Step 8.5.63C
8.5.68 If Applicable: Labeled vials. Labeled vials with QA provided labels. Attached a sample label.

8.5.69 If Applicable: Filled Vials. Aliquoted 1.0mL cell suspension, into appropriately sized cryovials. NOTE: Did not fill more than 10 vials of Excess TIL.
8.5.70 If Applicable: Filled Vials. Aliquoted residual volume into appropriately sized cryovial per SOP-00242. If volume is ≤0.5mL, add CS10 to vial until volume is 0.5mL.
8.5.71 If Applicable: Filled Vials. Filled one vial with 1.0mL of CS10 and label as "Blank".
8.5.72 If Applicable: Recorded number of vials filled. Recorded number of vials filled below, not including blank.
8.5.73 If Applicable: Environmental Monitoring. After processing, verified BSC and personnel monitoring had been performed

TIL Cryopreservation of Sample

8.5.74 If Applicable: Calculated Volume for Cryopreservation. Calculated the volume of cells required to obtain $1x10^7$ cells for cryopreservation.

Total Viable TIL required for cryopreservation	Viable Cell Concentration From Step 8.5.51A	Volume of Cells required for cryopreservation C=A÷B
A. $1x10^7$ cells	B. cells/mL	C. mL

8.5.75 If Applicable: Removed sample for Cryopreservation. In the BSC, using the appropriately sized syringe, removed the calculated volume (Step 8.5.74C) from the TIL Suspension transfer pack. Placed in appropriately sized conical tube and label as "Cryopreservation Sample $1x10^7$ cells," dated, and lot number. Placed a red cap (W3012845) on the TIL Suspension transfer pack.
8.5.76 If Applicable: Placed TIL in Incubator. Placed TIL Suspension Transfer Pack in incubator until needed.
8.5.77 If Applicable: Cryopreservation sample. Centrifuged the "Cryopreservation Sample $1x10^7$ cells" according to the following parameters: Speed: 350 x g, Time: 10:00 minutes, Temperature: Ambient, Brake: Full (9) Acceleration: Full (9). NOTE: Ensure proper units are set for speed and time on the centrifuge.
8.5.78 If Applicable: Observed conical tube. Recorded observations: Pellet observed? Supernatant is clear? *NOTE: If either answer is no, contact Area Management.
8.5.79 If Applicable: Added CS-10. In BSC, aseptically aspirate supernatant. Gently tap bottom of tube to resuspend cells in remaining fluid.
8.5.80 If Applicable: Added CS-10. Slowly added. 0.5mL of CS10. Recorded volume added.
8.5.81 If Applicable: Labeled vial. Labeled vial with QA issued label.
8.5.82 If Applicable: Filled Vials. Aliquoted resuspended volume into labeled cryovial.
8.5.83 If Applicable: Filled blank. Filled another vial with 0.5mL of CS10 and label as "Blank".
8.5.84 If Applicable: Recorded number of vials filled, not including "blank". Cryopreservation Sample Vials Filled at ~0.5mL

8.5.85 If Applicable: Environmental Monitoring. After processing, verify BSC and personnel monitoring have been performed.
8.5.86 Review Section 8.5

8.6 Day 11 - Feeder Cells

8.6.1 Obtained feeder cells. Obtained 3 bags of feeder cells with at least two different lot numbers from LN2 freezer. Kept cells on dry ice until ready to thaw. NOTE: Section 8.6 could be performed concurrently with Section 8.5.
8.6.2 Obtained feeder cells. Recorded feeder cell information. Confirmed that at least two different lots of feeder cells were obtained.
8.6.3 Prepared waterbath or Cryotherm. Prepared water bath or Cytotherm for Feeder Cell thaw.
8.6.4 Thawed Feeder Cells. Placed the Feeder Cell bags into individual zip top bags, based on Lot number, and thawed 37.0 ± 2.0°C water bath or cytotherm for ~3-5 minutes or until ice has just disappeared. Recorded thaw times below from timer.
8.6.5 Feeder cell harness preparation. Welded (per Process Note 5.11) 4S-4M60 to a CC2 Cell Connect (W3012820), replacing a single spike of the Cell Connect apparatus (B) with the 4-spike end of the 4S-4M60 manifold at (G). Welded H to G.
8.6.6 If applicable: Removed media from incubator. Removed the Feeder Cell CM2 Media transfer pack prepared in Step 8.4.34 from the incubator.
8.6.7 Attached media transfer pack Weld (per Process Note 5.11) the "Feeder Cell CM2 Media" transfer pack to a CC2 luer. NOTE: The bag will be attached to the side of the harness with the needless injection port.
8.6.8 Transfer harness. Transferred the assembly containing the Complete CM2 Day 11 Media into the BSC.
8.6.9 Pool thawed feeder cells. Within the BSC, pulled 10mL of air into a 100mL syringe. Used this to replace the 60mL syringe on the CC2.
8.6.10 Pool thawed feeder cells. Wiped each port on the feeder cell bags with an alcohol pad prior to removing the cover. Spike the three feeder bags using three of the spikes of the CC2. NOTE: Maintained constant pressure while turning the spike in one direction. Ensure to not puncture the side of the port.
8.6.11 Pool Thawed Feeder Cells. Opened the stopcock so that the line from the feeder cell bags is open and the line to the needless injection port is closed.
8.6.12 Pool Thawed Feeder Cells. Drew up the contents of the feeder cell bags into the syringe. All three bags drained at once. Once feeder cell bags had been drained, while maintaining pressure on the syringe, clamped off the line to the feeder cell bags.
8.6.13 Recorded volume of feeder cells. Did not detach syringe below. the syringe from the harness. Recorded the total volumeof feeder cells in the syringe.
8.6.14 Added feeder cells to transfer pack. Turned the stopcock so the line to the feeder cell bag is closed and the line to the media Transfer Pack was open. Ensured the line to media transfer pack is unclamped.
8.6.15 Added feeder cells to transfer pack Dispensed the feeder cells from the syringe into the "Feeder Cell CM2 Media" transfer pack. Clamped off the line to the transfer pack containing the feeder cells and leave the syringe attached to the harness.
8.6.16 Mixed pooled feeder cells. Massaged bag to mix the pooled feeder cells in the transfer pack.

8.6.17 Labeled transfer pack. Labeled bag as "Feeder Cell Suspension" and Lot number.
8.6.18 Calculated total volume in Transfer Pack Calculated the total volume of feeder cell suspension.
8.6.19 Removed cell count samples. Using a separate 3mL syringe for each sample, pulled 4x1.0mL cell count samples from Feeder Cell Suspension Transfer Pack using the needless injection port. Aliquoted each sample into the cryovials labeled in Step 8.4.27. NOTE: Wiped the needless injection port with a sterile alcohol pad (W3009488) and mixed Feeder Cell Suspension between each sampling for cell counts.
8.6.20 Performed Cell Counts. Performed cell counts and calculations utilizing NC-200 and Process Note 5.14. Diluted cell count samples by adding 0.5mL of cell suspension into 4.5mL of AIM-V media labelled with the lot number and "For Cell Count Dilutions". This will give a 1:10 dilution. Adjusted if necessary.
8.6.21 Recorded Cell Count. Sample volumes
8.6.22 Determine Multiplication Factor

Parameter	Formula	Result
Total cell count sample Volume	8.6.21A + 8.6.21B	C. µL
Multiplication Factor	C ÷ 8.6.21A	D.

8.6.23 Selected protocols and entered multiplication factors. Ensured the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered.
8.6.24 Recorded File Name, Viability and Cell Counts from Nucleoview.
8.6.25 Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	(8.6.24A + 8.6.24B) ÷ 2	E. %
Viable Cell Concentration	(8.6.24C + 8.6.24D) ÷ 2	F. cells/mL

8.6.26 Determined Upper and Lower Limit for counts.

Parameter	Formula	Result
Lower Limit	8.6.25F x 0.9	G. cells/mL
Upper Limit	8.6.25F x 1.1	H. cells/mL

8.6.27 Were both counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	8.6.24 C and D ≥ 8.6.26G	
Upper Limit	8.6.24 C and D ≤ 8.6.26H	

NOTE: If either result was "No" performed second set of counts in steps 8.6.28 – 8.6.35.

8.6.28 If Applicable: Performed cell counts Perform cell counts and calculations in utilizing NC-200 per SOP-00314 and Process Note 5.14 NOTE: Dilution could be adjusted according based off the expected concentration of cells.
8.6.29 If Applicable: Recorded Cell Count, sample volumes. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0.
8.6.30 If Applicable: Determined Multiplication Factor.

Parameter	Formula	Result
Total cell count sample Volume	8.6.29A + 8.6.29B	C. mL
Multiplication Factor	C ÷ 8.6.29A	D.

8.6.31 Select protocols and enter multiplication factors. Ensure the "Viable Cell Count Assay" protocol was selected, all multiplication factors, and sample and diluent volumes were entered. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0.
8.6.32 If Applicable: Recorded Cell Counts from Nucleoview
8.6.33 If Applicable: Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	(8.6.32A + 8.6.32B) ÷ 2	E. %
Viable Cell Concentration	(8.6.32C + 8.6.32D) ÷ 2	F. cells/mL

8.6.34 If Applicable: Determined Upper and Lower Limit for counts.

Parameter	Formula	Result
Lower Limit	8.6.33F x 0.9	G. cells/mL
Upper Limit	8.6.33F x 1.1	H. cells/mL

8.6.35 If Applicable: Were counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	8.6.32 C and D ≥ 8.6.34G	
Upper Limit	8.6.32 C and D ≤ 8.6.34H	

NOTE: If either result was "No," continued to step 8.6.36 to find a total Average Viable Cell Concentration and proceed with calculations.
8.6.36 If Applicable: Determined an average Viable Cell Concentration from all four counts performed.
8.6.37 Adjusted Volume of Feeder Cell Suspension. Calculated the adjusted volume of Feeder Cell suspension after removal of cell count samples. Total Feeder Cell Volume from Step 8.6.18C minus 4.0 ml removed.
8.6.38 Calculated Total Viable Feeder Cells.

Parameter	Formula	Result

Average Viable Cell Concentration*	**8.6.25 F* -or- 8.6.33 F* -or- 8.6.36 E***	**A.** cells/mL
Total Volume	**8.6.37 C**	**B.** mL
Total Viable Cells	**A x B**	**C.** cells

If Total Viable Cells are $< 5 \times 10^9$, proceed to Step 8.6.39. If Total Viable Cells are $\geq 5 \times 10^9$, proceed to Step 8.5.70.

8.6.39 If Applicable: Obtained additional Feeder Cells. Obtained an additional bag of feeder cells from LN2 freezer. Kept cells on dry ice until ready to thaw.

8.6.40 If Applicable: Obtained additional Feeder Cells. Recorded feeder cell information.

8.6.41 If Applicable: Thawed Additional Feeder Cells. Placed the 4th Feeder Cell bag into a zip top bag and thaw in a 37.0 ± 2.0°C water bath or cytotherm for ~3-5 minutes or until ice has just disappeared. Recorded thaw time.

8.6.42 If applicable: Pooled additional feeder cells. In the BSC, pulled 10 mL of air into a new 100mL syringe. Used this to replace the syringe on the harness.

8.6.43 If applicable: Pooled additional feeder cells Wiped the port of the feeder cell bag with an alcohol pad prior to removing the cover. Spiked the feeder cell bag using one of the remaining spikes of the harness prepared in Step 8.6.7 NOTE: Maintained constant pressure while turning the spike in one direction. Ensured to not puncture the side of the port.

8.6.44 If applicable: Pooled additional feeder cells. Opened the stopcock so that the line from the feeder cell bag was open and the line to the needless injection port was closed.

8.6.45 If applicable: Pooled additional feeder cells. Drew up the contents of the feeder cell bag into the syringe. Recorded volume.

8.6.46 If Applicable: Measured Volume. Measured the volume of the feeder cells in the syringe and recorded below (B). Calculated the new total volume of feeder cells.

Feeder Cell Volume from Step 8.6.37C	**Feeder Cell Volume from Step 8.6.45**	**Total Feeder Cell Volume C=A+B**
A. mL	**B.** mL	**C.** mL

8.6.47 If Applicable: Added Feeder Cells to Transfer Pack. Turned the stopcock so the line to the feeder cell bag was closed and the line to the "Feeder Cell Suspension" transfer pack was open. Ensured the line to the transfer pack was unclamped. Dispensed the feeder cells from the syringe into the "Feeder Cell Suspension" transfer pack. Clamped the line to the transfer pack and left the syringe attached to the harness.

8.6.48 If Applicable: Added Feeder Cells to Transfer Pack. Massaged bag to mix the pooled feeder cells in the Feeder Cell Suspension transfer pack.

8.6.49 If Applicable: Prepared dilutions. In the BSC, add 4.5mL of AIM-V Media that has been labelled with "For Cell Count Dilutions" and lot number

to four 15mL conical tubes. Label the tubes with the lot number and tube number (1-4). Labeled 4 cryovials "Additional Feeder" and vial number (1-4).
8.6.50 If Applicable: Prepared cell counts. Using a separate 3mL syringe for each sample, removed 4 x 1.0mL cell count samples from Feeder Cell Suspension transfer pack, using the needless injection port. Aliquoted each sample into cryovials labeled in Step 8.6.49. NOTE: Wiped the needless injection port with a sterile alcohol pad and mix Feeder Cell Suspension between each sampling for cell counts.
8.6.51 If Applicable: Performed Cell Counts. Performed cell counts and calculations utilizing NC-200 and Process Note 5.14. Diluted cell count samples by adding 0.5mL of cell suspension into 4.5mL of AIM-V media labelled with the lot number and "For Cell Count Dilutions". This will give a 1:10 dilution. Adjusted if necessary.
8.6.52 If Applicable: Recorded Cell Count sample volumes.
8.6.53 If Applicable: Determined Multiplication Factor

Parameter	Formula	Result
Total cell count sample Volume	8.6.52A + 8.6.52B	C. µL
Multiplication Factor	C ÷ 8.6.52A	D.

8.6.54 If Applicable: Selected protocols and entered multiplication factors. Ensured the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered.
8.6.55 If Applicable: Recorded File Name, Viability and Cell Counts from Nucleoview.
8.6.56 If Applicable: Determine the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	(8.6.55A + 8.6.55B) ÷ 2	E. %
Viable Cell Concentration	(8.6.55C + 8.6.55D) ÷ 2	F. cells/mL

8.6.57 If Applicable: Determine Upper and Lower Limit for counts.

Parameter	Formula	Result
Lower Limit	8.6.56F x 0.9	G. cells/mL
Upper Limit	8.6.56F x 1.1	H. cells/mL

Are both counts within acceptable limits? NOTE: If either result is "No" perform second set of counts in Steps 8.5.59 – 8.5.65
8.6.59 If Applicable: Performed cell counts. Performed cell counts and calculations in utilizing NC-200 and Process Note 5.14. NOTE: Dilution could be adjusted according based off the expected concentration of cells.
8.6.60 If Applicable: Recorded Cell Count sample volumes. NOTE: If no dilution was needed, entered "Sample [µL]" = 200, "Dilution [µL]" = 0
8.6.61 If Applicable: Determined Multiplication Factor.

Parameter	Formula	Result
Total cell count sample Volume	8.6.60A + 8.6.60B	C. µL

Multiplication Factor	**C ÷ 8.6.60A**	**D.**

8.6.62 If Applicable: Select protocols and enter multiplication factors. Ensured the "Viable Cell Count Assay" protocol has been selected, all multiplication factors, and sample and diluent volumes had been entered. NOTE: If no dilution was needed, entered "Sample [µL]" = 200, "Dilution [µL]" = 0

8.6.63 If Applicable: Recorded Cell Counts from Nucleoview.

8.6.64 If Applicable: Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	**(8.6.63A + 8.6.63B) ÷ 2**	**E.** %
Viable Cell Concentration	**(8.6.63C + 8.6.63D) ÷ 2**	**F.** cells/mL

8.6.65 If Applicable: Determined Upper and Lower Limit for counts

Parameter	Formula	Result
Lower Limit	**8.6.64F x 0.9**	**G.** cells/mL
Upper Limit	**8.6.64F x 1.1**	**H.** cells/mL

8.6.66 If Applicable: Were counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	**8.6.63** C and D ≥ **8.6.65G**	
Upper Limit	**8.6.63** C and D ≤ **8.6.65H**	

NOTE: If either result was "No," continue to Step 8.6.67 to find a total Average Viable Cell Concentration and proceeded with calculations.

8.6.67 If Applicable: Determined an average Viable Cell Concentration from all four counts performed.

8.6.68 If Applicable: Adjusted Volume of Feeder Cell Suspension. Calculated the adjusted volume of Feeder Cell suspension after removal of cell count samples. Total Feeder Cell Volume from Step 8.6.46C minus 4.0 mL removed.

8.6.69 If Applicable: Calculated Total Viable Feeder Cells.

Parameter	Formula	Result
Average Viable Cell Concentration*	**8.6.56 F*** **-or-** **8.6.64 F*** **-or-** **8.6.67 E***	**A.** cells/mL
Total Volume	**8.6.68 C**	**B.** mL
Total Viable Cells	**A x B**	**C.** cells

*Circled step reference used to determine Viable Cell Concentration.

8.6.70 Calculated Volume of Feeder Cells. Calculated the volume of Feeder Cell Suspension that was required to obtain $5x10^9$ viable feeder cells.

Number of Feeder Cells Required	Viable Cell Concentration from Step 8.6.38A* or Step 8.6.69A*	Volume of Feeder Cells = $5x10^9$ viable cells C=A÷B
A. $5x10^9$ Viable Cells	B. cells/mL	C. mL

*Circle applicable step

8.6.71 Calculated excess feeder cell volume. Calculated the volume of excess feeder cells to remove. Round down to nearest whole number.

Total Volume of Feeder Cells in Transfer Pack from Step 8.6.46C* or Step 8.6.68C*	Volume of Feeder Cells = $5x10^9$ viable cells from Step 8.6.70C	Volume of Excess Feeder Cells to remove. viable cells C=A-B
A. mL	B. mL	C. mL

*Circle applicable step

8.6.72 Removed excess feeder cells. In a new 100mL syringe, pulled up 10mL of air and attached the syringe to the harness.

8.6.73 Removed excess feeder cells. Opened the line to the "Feeder Cell Suspension" transfer pack. Using the syringe drew up the volume of feeder cells calculated in Step 8.6.71C plus an additional 10.0mL from the Transfer Pack into a 100mL syringe. Closed the line to the Feeder Cell Suspension transfer pack once the volume of feeder cells is removed. Did not remove final syringe. NOTE: Once a syringe has been filled, replaced it with a new syringe. Multiple syringes could be used to remove total volume. With each new syringe, pulled in 10mL of air.

8.6.74 Recorded volume. Recorded the total volume (including the additional 10mL) of feeder cells removed.

8.6.75 Added OKT3. In the BSC, using a 1.0mL syringe and 16G needle, drew up 0.15mL of OKT3.

8.6.76 Added OKT3. Aseptically removed the needle from the syringe and attach the syringe to the needless injection port. Injected the OKT3.

8.6.77 Added OKT3. Opened the stopcock to the "Feeder Cell Suspension" transfer pack and added 10mL of feeder cells removed in Step 8.6.73 to flush OKT3 through the line.

8.6.78 Added OKT3. Turned the syringe upside down and push air through to clear the line to the Feeder Cell Suspension transfer pack.

8.6.79 Added OKT3. Left the remaining feeder cell suspension in the syringe. Closed all clamps and remove the harness from the BSC.

8.6.80 Heat Sealed. Heat sealed (per Process Note 5.12) the Feeder Cell Suspension transfer pack, leaving enough tubing to weld. Discarded the harness.

8.6.81 Review Section 8.6

8.7 Day 11 G-Rex Fill and Seed

8.7.1 Set up G-Rex500MCS. Outside the BSC, removed a G-Rex500MCS from packaging and inspected the flask for any cracks or kinks in the tubing. Ensured all luer connections and closures were tight. Closed all clamps on the

G-Rex500MCS lines except for the vent filter line. Using a marker drew a line at the 4.5L gradation.

8.7.2 Removed media from incubator. Removed the "Complete CM2 Day 11 Media", prepared in Step 8.4.35, from theincubator.

8.7.3 Prepared to pump media. Welded (per Process Note 5.11) the red line of the G-Rex500MCS to the repeater pump transfer set attached to the complete CM2 Day 11 Media.

8.7.4 Prepare to pump media. Hung the "Complete CM2 Day 11 Media" bag on an IV pole. Fed the pump tubing through the Baxa pump.

8.7.5 Pumped media into G-Rex500MCS. Set the Baxa pump to "High" and "9". Pumped 4.5L of media into the G-Rex500MCS, filling to the line marked on the flask in Step 8.7.1.

8.7.6 Heat sealed. Heat sealed the red line (per Process Note 5.12) of the G-Rex500MCS near the weld created in Step 8.7.3.

8.7.7 Labeled Flask. Labeled the flask with the Attach a sample "Day 11 QA provided in-process "Day 11" label.

8.7.8 If applicable: Incubated flask. Held flask in incubator while waiting to seed with TIL.

8.7.9 Welded the Feeder Cell: Suspension transfer pack to the flask . Sterile welded (per Process Note 5.11) the red line of the G-Rex500MCS to the "Feeder Cell Suspension" transfer pack.

8.7.10 Added Feeder Cells to G-Rex500MCS. Opened all clamps between Feeder Cell Suspension and G-Rex500MCS and added Feeder Cell Suspension to flask by gravity feed. Ensured the line has been completely cleared.

8.7.11 Heat sealed. Heat sealed (per Process Note 5.12) the red line near the weld created in Step 8.7.9.

8.7.12 Welded the TIL Suspension transfer pack to the flask. Sterile weld (per Process Note 5.11) the red line of the G-Rex500MCS to the "TIL Suspension" transfer pack.

8.7.13 Added TIL to G-Rex500MCS. Opened all clamps between TIL Suspension and G-Rex500MCS and added TIL Suspension to flask by gravity feed. Ensured the line has been completely cleared.

8.7.14 Heat sealed. Heat sealed (per process note 5.12) the red line near the weld created in Step 8.7.12 to remove the TIL suspension bag.

8.7.15 Incubated G-Rex500MCS. Checked that all clamps on the G-Rex500MCS were closed except the large filter line and place in the incubator. Incubator parameters: Temperature LED Display: 37.0±2.0 °C, CO2 Percentage: 5.0±1.5 %CO2.

8.7.16 Calculated incubation window. Performed calculations to determine the proper time to remove G-Rex500MCS from incubator on Day 16. Time of incubation (Step 8.7.15). Lower limit: Time of incubation + 108 hours. Upper limit: Time of incubation + 132 hours.

8.7.17 Environmental Monitoring. After processing, verified BSC and personnel monitoring had been performed.

8.7.18 Submit Samples. Submit samples to Login.

8.7.19 Review Section 8.7

8.8 Day 11 Excess TIL Cryopreservation

8.8.1 If Applicable: Froze Excess TIL Vials. Verified the CRF has been set up prior to freeze. Perform Cryopreservation.

8.8.2 If Applicable: Started CRF. Recorded the total number of vials placed into the CRF (not including blank). Verify number of vials transferred into the CRF matches total number of vials prepared in Step 8.5.72 or Step 8.5.84 Step 8.5.72C or Step 8.5.84
8.8.3 If applicable: Initiated automated portion of the freezing profile. Recorded START TIME for the initiation of the automated portion of the freezing profile.
8.8.4 If Applicable: Transferred vials from Controlled Rate Freezer to the appropriate storage. Upon completion of freeze, transfer vials from CRF to the appropriate storage container.
8.8.5 If applicable: Transferred vials to appropriate storage. Recorded storage location in LN2.
8.8.6 Review Section 8.8
8.9 Day 16 Media Preparation
8.9.1 Pre-warmed AIM-V Media. Removed three CTS AIM V 10L media bags from 2-8°C at least 12 hours prior to use and place at room temperature protected from light. Verify each bag is within expiry. Labeled each bag with Bag Number (1-3), lot number, date, and "warming start time HHMM". Record warming start time and date.
8.9.2 Calculated time Media from step 8.9.1 was warmed. Calculated the warming time of media bags 1, 2, and 3 from step 8.9.1. Ensured all bags have been warmed for a duration between 12 and 24 hours.
8.9.3 Checked room sanitization, line clearance, and materials. Confirmed room sanitization, line clearance, and materials.
8.9.4 Ensured completion of pre-processing table.
8.9.5 Environmental Monitoring. Prior to processing, ensured pre-process environmental monitoring had been initiated.
8.9.6 Setup 10L Labtainer for Supernatant. In the BSC attached the larger diameter end of a fluid pump transfer set to one of the female ports of a 10L Labtainer bag using the Luer connectors.
8.9.7 Setup 10L Labtainer for Supernatant Label as "Supernatant" and Lot number.
8.9.8 Setup 10L Labtainer for Supernatant Ensure all clamps were closed prior to removing from the BSC. NOTE: Supernatant bag was used during TIL Harvest (Section 8.10), which may be performed concurrently with media preparation.
8.9.9 Thawed IL-2. Thawed 5x1.1mL aliquots of IL-2 ($6x10^6$ IU/mL) (BR71424) per bag of CTS AIM V media until all ice had melted. Recorded IL-2 Lot number and Expiry. Attached IL-2 labels.
8.9.10 Aliquoted GlutaMax. In BSC, aliquoted 100.0mL of Glutamax into an appropriately sized receiver. Recorded the volume added to each receiver NOTE: Initially prepared one bag of AIM-V media following Step 8.9.10 - Step 8.9.28. Additional bags required were determined in Step 8.10.59.
8.9.11 Labeled receivers. Labeled each receiver as "GlutaMax."
8.9.12 Added IL-2 to GlutaMax. Using a micropipette, added 5.0mL of IL-2 to each GlutaMax receiver. Ensured to rinse the tip per process note 5.18 and used a new pipette tip for each mL added. Recorded volume added to each Glutamax receiver below.
8.9.13 Labeled receivers. Labeled each receiver as "GlutaMax + IL-2" and receiver number.

8.9.14 Prepared CTS AIM V media bag for formulation. Ensured CTS AIM V 10L media bag (W3012717) was warmed at room temperature and protected from light for 12-24 hours prior to use. Recorded end incubation time in Step 8.9.2.
8.9.15 Prepared CTS AIM V media bag for formulation. In the BSC, closed clamp on a 4" plasma transfer set, then connected to the bag using the spike ports. NOTE: Maintained constant pressure while turning the spike in one direction. Ensured to not puncture the side of the port.
8.9.16 Prepared CTS AIM V media bag for formulation. Connected the larger diameter end of a repeater pump fluid transfer set to the 4" plasma transfer set via luer.
8.9.17 Stage Baxa Pump. Stage Baxa pump next to BSC. Removed pump tubing section of repeater pump fluid transfer set from BSC and installed in repeater pump.
8.9.18 Prepared to formulate media. In BSC, removed syringe from Pumpmatic Liquid-Dispensing System (PLDS) and discarded. NOTE: Ensured to not compromise the sterility of the PLDS pipette.
8.9.19 Prepared to formulate media. Connected PLDS pipette to smaller diameter end of repeater pump fluid transfer set via luer connection and placed pipette tip in "GlutaMax + IL-2" prepared in Step 8.9.13 for aspiration
Open all clamps between receiver and 10L bag.
8.9.20 Pumped GlutaMax +IL-2 into bag. Set the pump speed to "Medium" and "3" and pump all "GlutaMax + IL-2" into 10L CTS AIM V media bag. Once no solution remains, clear line and stop pump. Recorded the volume of GlutaMax containing IL-2 added to each Aim V bag below.Recorded/
8.9.21 Removed PLDS. Ensured all clamps were closed, and removed the PLDS pipette from the repeater pump fluid transfer set. Removed repeater pump fluid transfer set and red cap the 4" plasma transfer set.
8.9.22 Labeled Bags. Labeled each bag of "Complete CM4 Day 16 media" prepared.
8.9.23 Removed Media Retain per Sample Plan. Using a 30mL syringe, removed 20.0mL of "Complete CM4 Day 16 media" by attaching syringe to the 4" plasma transfer set and dispensed sample into a 50mL conical tube. NOTE: Only removed the Media Retain Sample from the first bag of media prepared. NOTE: Ensure 4" plasma transfer set was either clamped or red capped after removal of syringe.
8.9.24 Attached new repeater pump fluid transfer set. Attached the larger diameter end of a new fluid pump transfer set onto the 4" plasma transfer set that was connected to the "Complete CM4 Day 16 media" bag.
8.9.25 Labeled and stored sample. Labeled with sample plan inventory label and stored media retain sample at 2-8°C until submitted to Login for testing per Sample Plan.
8.9.26 Signed for Sampling. Ensured that LIMS sample plan sheet was completed for removal of the sample.
8.9.27 Monitor Incubator. Monitored Incubator. If applicable, per Step 8.9.10, monitor for additional bags prepared. Incubator parameters: Temperature LED Display: 37.0±2.0 °C, CO2 Percentage: 5.0±1.5 %CO2.
8.9.28 Warmed Complete CM4 Day 16 Media. Warmed the first bag of Complete CM4 Day 16 Media in incubator for ≥ 30 minutes until ready for use. If applicable, per Step 8.10.59, warmed additional bags.

8.9.29 Prepared Dilutions. In the BSC, added 4.5mL of AIM-V Media that had been labelled with Batch record Lot Number and "For Cell Count Dilutions" to each 4x15mL conical tube. Labeled the conical tubes with the lot number and tube number (1-4). Labeled 4 cryovials with vial number (1-4). Kept vials under BSC to be used in Step 8.10.31.
8.9.30 Reviewed Section 8.9
8.10 Day 16 REP Spilt
8.10.1 Pre-processing table.
8.10.2 Monitored Incubator. Monitored Incubator. Incubator parameters: Temperature LED Display: 37.0±2.0 ºC, CO2 Percentage: 5.0±1.5 %CO2
8.10.3 Removed G-Rex500MCS from Incubator. Performed check below to ensure incubation parameters are met before removing G-Rex500MCS from incubator.

Lower limit from Step 8.7.16B (DDMMMYY HHMM)	Upper limit from Step 8.7.16C (DDMMMYY HHMM)	Time of Removal from incubator (DDMMMYY HHMM)	Is 8.7.16B < Time of Removal from incubator < Step 8.7.16C Yes/No*

Removed G-Rex500MCS from the incubator.
8.10.4 Setup 1L Transfer Pack. Heat sealed a 1L transfer pack (W3006645) per Processed Note 5.12, leaving ~12" of line.
8.10.5 Prepared 1L Transfer Pack. Labeled 1L transfer pack as TIL Suspension.
8.10.6 Weighed 1L Transfer Pack Place 1L transfer pack, including the entire line, on a scale and record dry weight.
8.10.7 GatheRex Setup. Sterile welded (per Process Note 5.11) the red media removal line from the G-Rex500MCS to the repeater pump transfer set on the 10L labtainer bag "Supernatant" prepared in Step 8.9.8. Sterile welded the clear cell removal line from the G-Rex500MCS to the TIL Suspension transfer pack prepared in Step 8.10.5.
8.10.8 GatheRex Setup. Placed G-Rex500MCS flask on the left side of the GatheRex. Placed the supernatant labtainer bag and TIL suspension transfer pack to the right side.
8.10.9 GatheRex Setup. Installed the red media removal line from the G-Rex500MCS to the top clamp (marked with a red line) and tubing guides on the GatheRex. Installed the clear harvest line from the G-Rex500MCS to the bottom clamp (marked with a blue line) and tubing guides on the GatheRex.
8.10.10 GatheRex Setup. Attached the gas line from the GatheRex to the sterile filter of the G-Rex500 MCS. NOTE: Before removing the supernatant from the G-Rex500MCS, ensured all clamps on the cell removal lines were closed.
8.10.11 Volume Reduction of G-Rex500MCS. Transferred ~4.5L of culture supernatant from the G-Rex500MCS to the 10L Labtainer per SOP-01777. Visually inspect G-Rex500MCS to ensure flask as level and media had been reduced to the end of the aspirating dip tube. NOTE: If the GatheRex stops prematurely, it could be restarted by pressing the button with the arrow pointing to the right again.
8.10.12 Prepared flask for TIL Harvest. After removal of the supernatant, closed all clamps to the red line.

8.10.13 Initiation of TIL Harvest. Recorded the start time of the TIL harvest.
8.10.14 Initiation of TIL Harvest. Vigorously tap flask and swirl media to release cells. Performed an inspection of the flask to ensure all cells have detached. NOTE: Contact area management if cells did not detach.
8.10.15 Initiation of TIL Harvest. Tilted the flask to ensure hose is at the edge of the flask. Note: If the cell collection straw is not at the junction of the wall and bottom membrane, rapping the flask while tilted at a 450 angle is usually sufficient to properly position the straw.
8.10.16 TIL Harvest. Released all clamps leading to the TIL suspension transfer pack.
8.10.17 TIL Harvest. Using the GatheRex transferred the cell suspension into the TIL Suspension transfer pack. NOTE: Be sure to maintain the tilted edge until all cells and media are collected.
8.10.18 TIL Harvest. Inspected membrane for adherent cells.
8.10.19 Rinse flask membrane. Rinse the bottom of the G-Rex500MCS. Cover ~1/4 of gas exchange membrane with rinse media.
8.10.20 Closed clamps on G-Rex500MCS. Ensured all clamps are closed on the G-Rex500MCS.
8.10.21 Heat sealed. Heat sealed (per Process Note 5.12) the Transfer Pack containing the TIL as close to the weld as possible so that the overall tubing length remained approximately the same.
8.10.22 Heat sealed. Heat sealed the 10L Labtainer containing the supernatant (per Process Note 5.12) and passed into the BSC for sample collection in Step 8.10.25.
8.10.23 Calculated volume of TIL suspension. Recorded weight of Transfer Pack with cell suspension and calculate the volume suspension.
8.10.24 Prepared transfer pack for sample removal. Welded (per Process Note 5.11) a 4" Plasma Transfer Set, to the TIL Suspension transfer pack from Step 8.10.21, leaving the female luer end attached as close to the bag as possible.
8.10.25 Removed testing samples from cell supernatant. In the BSC, remove 10.0 mL of supernatant from 10L labtainer using female luer port and appropriately sized syringe. Placed into a 15mL conical tube and label as "BacT" Retain the tube for BacT sample in Step 8.10.28.
8.10.26 Removed testing samples from cell supernatant. Using a separate syringe, removed 10.0 mL of supernatant and placed into a 15mL conical tube. Retained the tube for mycoplasma sample for use in Step 8.10.32. Labeled tube as "Mycoplasma diluent"
8.10.27 Closed supernatant bag. Placed a red cap on the luer port to close the bag, and pass out of BSC.
8.10.28 Sterility & BacT Testing Sampling. In the BSC, removed a 1.0mL sample from the 15 mL conical labeled BacT prepared in Step 8.10.25 using an appropriately sized syringe and inoculate the anaerobic bottle. Repeat the above for the aerobic bottle. NOTE: This step may be performed out of sequence.
8.10.29 Labeled and store samples. Labeled with sample plan inventory label and store BacT sample at room temperature, protected from light, until submitted to Login for testing per Sample Plan. NOTE: Did not cover barcode on bottle with label.

8.10.30 Signed for Sampling. Ensured that LIMS sample plan sheet is completed for removal of the sample.

8.10.31 Removed Cell Count Samples. In the BSC, using separate 3mL syringes for each sample, removed 4x1.0 mL cell count samples from "TIL Suspension" transfer pack using the luer connection. Placed samples in cryovials prepared in Step 8.9.29.

8.10.32 Removed Mycoplasma Samples. Using a 3mL syringe, removed 1.0 mL from TIL Suspension transfer pack and place into 15 mL conical labeled "Mycoplasma diluent" prepared in Step 8.10.26.

8.10.33 Label and store sample. Labeled with sample plan inventory label and stored Mycoplasma sample at 2-8ºC until submitted to Login for testing per Sample Plan.

8.10.34 Signed for Sampling. Ensured that LIMS sample plan sheet was completed for removal of the sample.

8.10.35 Prepared Transfer Pack for Seeding. In the BSC, attached the large diameter tubing end of a Repeater Pump Fluid Transfer Set to the Luer adapter on the transfer pack containing the TIL. Clamped the line close to the transfer pack using a hemostat. Placed a red cap onto the end of the transfer set.

8.10.36 Placed TIL in Incubator. Removed cell suspension from the BSC and place in incubator until needed. Recorded time.

8.10.37 Performed Cell Counts. Performed cell counts and calculations utilizing NC-200 and Process Note 5.14. Diluted cell count samples initially by adding 0.5mL of cell suspension into 4.5mL of AIM-V media prepared in Step 8.9.29. This gave a 1:10 dilution.

8.10.38 Recorded Cell Count sample volumes

8.10.39 Determined Multiplication Factor.

Parameter	Formula	Result
Total cell count sample Volume	8.10.38A + 8.10.38B	C. µL
Multiplication Factor	C ÷ 8.10.38A	D.

8.10.40 Selected protocols and enter multiplication factors. Ensured the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered.

8.10.41 Recorded File Name, Viability and Cell Counts from Nucleoview.

8.10.42 Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	(8.10.41A + 8.10.41B) ÷ 2	E. %
Viable Cell Concentration	(8.10.41C + 8.10.41D) ÷ 2	F. cells/mL

8.10.43 Determined Upper and Lower Limit for counts.

Parameter	Formula	Result

Lower Limit	**8.10.42F x 0.9**	**G.** cells/mL
Upper Limit	**8.10.42F x 1.1**	**H.** cells/mL

8.10.44 Were both counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	**8.10.41**C and D ≥ 8.**10.43G**	
Upper Limit	**8.10.41** C and D ≤ 8.**10.43H**	

8.10.45 If Applicable: Performed cell counts. Performed cell counts and calculations in utilizing NC-200 and Process Note 5.14. NOTE: Dilution may be adjusted according based off the expected concentration of cells.

8.10.46 If Applicable: Recorded Cell Count sample volumes. NOTE: If no dilution was needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0

8.10.47 If Applicable: Determined Multiplication Factor.

Parameter	Formula	Result
Total cell count sample Volume	**8.10.46A + 8.10.46B**	**C.** mL
Multiplication Factor	**C ÷ 8.10.46A**	**D.**

8.10.48 If Applicable: Select protocols and enter multiplication factors. Ensure the "Viable Cell Count Assay" protocol has been selected, all multiplication factors, and sample and diluent volumes have been entered. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0

8.10.49 If Applicable: Recorded Cell Counts from Nucleoview

8.10.50 If Applicable: Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	**(8.10.49A + 8.10.49B) ÷ 2**	**E.** %
Viable Cell Concentration	**(8.10.49C + 8.10.49D) ÷ 2**	**F.** cells/mL

8.10.51 If Applicable: Determined Upper and Lower Limit for counts.

Parameter	Formula	Result
Lower Limit	**8.10.50F x 0.9**	**G.** cells/mL
Upper Limit	**8.10.50F x 1.1**	**H.** cells/mL

8.10.52 If Applicable: Were counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	**8.10.49** C and D ≥ 8.**10.51G**	
Upper Limit	**8.10.49** C and D ≤ 8.**10.51H**	

NOTE: If either result is "No" continue to Step 8.10.53 to determine an average of all cell counts collected.

8.10.53 If Applicable: Determined an average Viable Cell Concentration from all four counts performed.

8.10.54 Adjusted Volume of TIL Suspension. Calculated the adjusted volume of TIL suspension after removal of cell count samples. Total TIL Cell Volume from Step 8.10.23C minus 5.0 mL removed for testing.

8.10.55 Calculated Total Viable TIL Cells.

Parameter	Formula	Result
Average Viable Cell Concentration*	8.10.42 F* -or- 8.10.50 F* -or- 8.10.53E*	A. cells/mL
Total Volume	8.10.54 C	B. mL
Total Viable Cells	A x B	C. cells

8.10.56 Calculated flasks for subculture. Calculated the total number of flasks to seed. NOTE: Rounded the number of G-Rex500MCS flasks to see up to the neared whole number.

Total Viable Cell Count from Step 8.10.55C A	Target Cells Required per Flask B	Number of G-Rex500MCS Flasks to Seed C= A÷B
cells	$1.0x10^9$ cells/flask	flasks

NOTE: The maximum number of G-Rex500MCS flasks to seed was five. If the calculated number of flasks to seed exceeded five, only five were seeded USING THE ENTIRE VOLUME OF CELL SUSPENSION AVAILABLE

8.10.57 Calculate number of flasks for subculture

Criteria	Yes/No
Number of G-Rex500MCS Flasks to Seed Step 8.10.56C ≤ 5 If yes, seed number of flasks calculated in Step 8.10.58.	
Number of G-Rex500MCS Flasks to Seed Step 8.10.56C > 5 If yes, seed 5 flasks with ALL available cells.	

8.10.58 QA Review of Cell Count calculations performed in steps 8.10.38 – 8.10.57.

8.10.59 Determined number of additional media bags needed. Calculated the number of media bags required in addition to the bag prepared in Step 8.9.28.

Number of G-Rex500MCS Flasks to Seed (Step 8.10.56C) A	Number of Media Bag Required B=A÷2*	Number of Bags Prepared in Step 8.9.22 C	Number of Additional Bags to Prepare D=B-C
		1	

*Round the number of media bags required up to the next whole number.

8.10.60 If Applicable: Prepared additional media. Prepared one 10L bag of "CM4 Day 16 Media" for every two G-Rex-500M flask needed

calculated in Step 8.10.59D. Proceeded to Step 8.10.62 and seeded the first GREX-500M flask(s) while additional media is prepared and warmed.

8.10.61 If Applicable: Prepared additional media bags. Prepared and warmed the calculated number of additional media bags determined in Step 8.10.59D, repeating Step 8.9.10 - Step 8.9.28.

8.10.62 Filled G-Rex500MCS. Opened a G-Rex500MCS on the benchtop and inspected for cracks in the vessel or kinks in the tubing. Ensured all luer connections and closures were tight. Made a mark at the 4500mL line on the outside of the flask with a marker. Closed all clamps on the G-Rex500MCS except the large filter line.

8.10.63 Filled G-Rex500MCS. Sterile welded (per Process Note 5.11) the red media line of a G-Rex500MCS to the fluid transfer set on the media bag prepared in Step 8.9.28.

8.10.64 Prepared to pump media. Hung "CM4 Day 16 Media" on an IV pole. Fed the pump tubing through the Baxa pump.

8.10.65 Pump media into G-Rex500MCS. Set the Baxa pump on "High" and "9" and pump 4500mL of media into the flask. Pumped 4.5L of "CM4 Day 16 Media" into the G-Rex500MCS, filling to the line marked on the flask in Step 8.10.62. Once 4.5L of media had been transferred, stopped the pump.

8.10.66 Heat Sealed. Heat sealed (per Process Note 5.12) the red media line of G-Rex500MCS, near the weld created in Step 8.10.63, removing the media bag.

8.10.67 Repeated Fill. Repeat Steps 8.10.62-8.10.66 for each flask calculated in Step 8.10.56C as media is warmed and prepared for use. NOTE: Multiple flasks may be filled at the same time using gravity fill or multiple pumps. NOTE: Fill only two flasks per bag of media.

8.10.68 Recorded and labelled flask(s) filled. Labeled each flask alphabetically as it is filled and with QA provided in-process "Day 16" labels.

8.10.69 Sample Labeled. Attached a sample "Day 16" label below.

8.10.70 If applicable: Incubated flask. Held flask in incubator while waiting to seed with TIL.

8.10.71 Verified Number of Flasks Filled. Recorded the total number of flasks filled.

8.10.72 Calculated volume of cell suspension to add. Calculated the target volume of TIL suspension to add to the new G-Rex500MCS flasks.

Total Volume of TIL suspension from Step 8.10.54C A	Number of flask(s) filled from Step 8.10.71	Target Volume of cell suspension to transfer to each flask C= A÷B
mL		mL

8.10.56C exceeds five only five will be seeded, USING THE ENTIRE VOLUME OF CELL SUSPENSION.

8.10.73 Prepared Flasks for Seeding. Removed G-Rex500MCS from Step 8.10.70 from the incubator.

8.10.74 Prepared for pumping. Closed all clamps on G-Rex500MCS except large filter line. Fed the pump tubing through the Baxa pump.

8.10.75 Removed TIL from incubator. Removed "TIL Suspension" transfer pack from the incubator and record incubation end time in Step 8.10.36.

8.10.76 Prepared cell suspension for seeding. Sterile welded (per Process Note 5.11) "TIL Suspension" transfer pack from Step 8.10.75 to pump inlet line.

8.10.77 Tared scale. Placed TIL suspension bag on a scale. Primed the line from the TIL suspension bag to the weld using the Baxa pump set to "Low" and "2". Tared the scale.

8.10.78 Seeded flask with TIL Suspension. Set Baxa pump to "Medium" and "5". Pump the volume of TIL suspension calculated in Step 8.10.72C into flask. Record the volume of TIL Suspension added to each flask.

8.10.79 Heat sealed. Heat sealed (per Process Note 5.12) the "TIL Suspension" transfer pack, leaving enough tubing to weld on the next flask. Used the line stripper to clear the residual TIL suspension in the G-Rex flask line into the vessel.

8.10.80 Filled remaining flasks. Between each flask seeded, ensured to mix "TIL Suspension" transfer pack and repeat Steps 8.10.76-8.10.79 to seed all remaining flaks. Filled flask(s) in alphabetical order.

8.10.81 Monitored Incubator. NOTE: If flasks must be split among two incubators, ensure to monitor both. Incubator parameters: Temperature LED Display: 37.0±2.0 ºC, CO2 Percentage: 5.0±1.5 %CO2.

8.10.82 Incubated Flasks. Recorded the time each flask is placed in the incubator.

8.10.83 Calculated incubation window. Performed calculations below to determine the time range to remove G-Rex500MCS from incubator on Day 22.

Flask	8.10.83A Time of incubation (Step 8.10.82) (DDMMMYY HHMM)	8.10.83B Lower limit: Time of incubation + 132 hours (DDMMMYY HHMM)	8.10.83C Upper limit: Time of incubation + 156 hours (DDMMMYY HHMM)

8.10.84 Environmental Monitoring. After processing, verified BSC and personnel monitoring had been performed.

8.10.85 Sample Submission. Ensured all Day 16 Samples were submitted to Login.

8.10.86 Reviewed Section 8.10.

8.11 Day 22 Wash Buffer Preparation

8.11.1 Checked room sanitization, line clearance, and materials.

8.11.2 Ensured completion of pre-processing checklist.

8.11.3 Environmental monitoring. Prior to processing, ensured pre-process environmental monitoring had been performed.

8.11.4 Prepared 10 L Labtainer Bag In BSC, attach a 4" plasma transfer set to a 10L Labtainer Bag via luer connection.

8.11.5 Prepared 10 L Labtainer Bag Label as "Supernatant", lot number, and initial/date.

8.11.6 Prepared 10 L Labtainer Bag. Closed all clamps before transferring out of the BSC. NOTE: Prepared one 10L Labtainer Bag for every two G-Rex500MCS flasks to be harvested. NOTE: Supernatant bag(s) were used in Section 8.12, which could be run concurrently with Section 8.11.

8.11.7 Welded fluid transfer set. Outside the BSC, closed all clamps on 4S-4M60. Welded (per Process Note 5.11) repeater fluid transfer set to one of the male luer ends of 4S-4M60.
8.11.8 Passed materials into the BSC. Passed Plasmalyte-A and Human Albumin 25% into the BSC. Pass the 4S-4M60 and repeater fluid transfer set assembly into the BSC.

Component Description	Amount Needed
Plasmalyte-A	3000.0 mL
Human Albumin 25%	120.0 mL
4S-4M60 with Repeater Fluid Transfer Set	1 Apparatus Step 8.11.7

8.11.9 Pumped Plasmalyte into 3000mL bag. Spiked three bags of Plasmalyte-A to the 4S-4M60 Connector set. NOTE: Wipe the port cover with an alcohol swab (W3009488) prior to removing. NOTE: Maintain constant pressure while turning the spike in one direction. Ensure to not puncture the side of the port.
8.11.10 Pumped Plasmalyte into 3000mL bag. Connected an Origen 3000mL collection bag via luer connection to the larger diameter end of the repeater pump transfer set.
8.11.11 Pumped Plasmalyte into 3000mL bag. Closed clamps on the unused lines of the 3000mL Origen Bag.
8.11.12 Pumped Plasmalyte into 3000mL bag. Staged the Baxa pump next to the BSC. Fed the transfer set tubing through the Baxa pump situated outside of the BSC. Set pump to “High” and “9”.
8.11.13 Pumped Plasmalyte into 3000mL bag. Opened all clamps from the Plasmalyte-A to the 3000mL Origen Bag.
8.11.14 Pump Plasmalyte into 3000mL bag. Pumped all of the Plasmalyte-A into the 3000 mL Origen bag. Once all the Plasmalyte-A had been transferred, stopped the pump.
8.11.15 Pumped Plasmalyte into 3000mL bag. If necessary, removed air from 3000mL Origen bag by reversing the pump and manipulating the position of the bag.
8.11.16 Pumped Plasmalyte into 3000mL bag. Closed all clamps. Remove the 3000mL bag from the repeater pump fluid transfer set via luer connection and placed a red cap (W3012845) on the line to the bag.
8.11.17 Added Human Albumin 25% to 3000mL Bag. Opened vented mini spike. Without compromising sterility of spike, ensured blue cap is securely fastened.
8.11.18 Added Human Albumin 25% to 3000mL Bag. Spiked the septum of a Human Albumin 25% bottle with the vented mini spike. NOTE: Ensured to not compromise the sterility of the spike.
8.11.19 Added Human Albumin 25% to 3000mL Bag. Repeated Step 8.11.17 – Step 8.11.18 two times for a total of three (3) spiked Human Albumin 25% bottles.

8.11.20 Added Human Albumin 25% to 3000mL Bag. Removed the blue cap from one vented mini spike and attach a 60mL syringe to the Human Serum Albumin 25% bottle.
8.11.21 Added Human Albumin 25% to 3000mL Bag. Draw up 60mL of Human Serum Albumin 25%. NOTE: It may be necessary to use more than one bottle of Human Serum Albumin 25%. If necessary, disconnect the syringe from the vented mini spike and connect it to the next vented mini spike in a Human Serum Albumin 25% bottle. Do not remove vented mini spike from the Human Serum Albumin 25% bottle.
8.11.22 Added Human Albumin 25% to 3000mL Bag. Once 60mL has been obtained, remove the syringe from the vented mini spike.
8.11.23 Added Human Albumin 25% to 3000mL Bag. Attach syringe to needleless injection port on 3000mL Origen bag filled with Plasmalyte-A in Step 8.11.16. Dispensed all of the Human Albumin 25%. NOTE: Wiped needless injection port with an alcohol pad before each use.
8.11.24 Added Human Albumin 25% to 3000mL Bag. Repeated Step 8.11.20 - Step 8.11.23 to obtain a final volume of 120.0 mL of Human Albumin 25%.
8.11.25 Mixed Bag. Gently mixed the bag after all of the Human Albumin 25% had been added.
8.11.26 Labeled Bag. Labeled as "LOVOWash Buffer" and lot number, and assign a 24 hour expiry.
8.11.27 Prepared IL-2 Diluent. Using a 10mL syringe, removed 5.0 mL of LOVO Wash Buffer using the needleless injection port on the LOVO Wash Buffer bag. Dispensed LOVO wash buffer into a 50mL conical tube and label as "IL-2 Diluent" and the lot number. NOTE: Wiped the needless injection port with an alcohol pad before each use.
8.11.28 CRF Blank Bag LOVO Wash Buffer Aliquotted. Using a 100mL syringe, drew up 70.0 mL of LOVO Wash Buffer from the needleless injection port. NOTE: Wiped the needless injection port with an alcohol pad before each use.
8.11.29 CRF Blank Bag LOVO Wash Buffer Aliquotted. Placed a red cap on the syringe and label as "blank cryo bag" and lot number. NOTE: Held the syringe at room temp until needed in Step 8.14.3
8.11.30 Completed Wash Buffer Prep. Closed all clamps on the LOVO Wash Buffer bag.
8.11.31 Thawed IL-2. Thawed one 1.1mL of IL-2 ($6x10^6$ IU/mL)), until all ice has melted. Record IL-2 Lot number and Expiry. NOTE: Ensured IL-2 label is attached.
8.11.32 IL-2 Preparation. Added 50µL IL-2 stock ($6x10^6$ IU/mL) to the 50mL conical tube labeled "IL-2 Diluent."
8.11.33 IL-2 Preparation. Relabeled conical as "IL-2 $6x10^4$", the date, lot number, and 24 hour expiry. Cap and store at 2-8°C.
8.11.34 Cryopreservation Prep. Placed 5 cryo-cassettes at 2-8°C to precondition them for final product cryopreservation.
8.11.35 Prepared Cell Count Dilutions. In the BSC, added 4.5mL of AIM-V Media that has been labelled with lot number and "For Cell Count Dilutions" to 4 separate 15mL conical tubes. Labeled the tubes with the batch record lot number and tube number (1-4). Set aside for use in Step 8.12.34

8.11.36 Prepared Cell Counts. Labeled 4 cryovials with vial number (1-4). Kept vials under BSC to be used in Step 8.12.33.

8.11.37 Reviewed Section 8.11

8.12 Day 22 TIL Harvest

8.12.1 Monitor Incubator. Monitored the incubator. Incubator Parameters Temperature LED display: 37 ± 2.0°C, CO2 Percentage: 5%±1.5%. NOTE: Section 8.12 could be run concurrently with Section 8.11.

8.12.2 Removed G-Rex500MCS Flasks from Incubator. Performed check below to ensure incubation parameters were met before removing G-Rex500MCS from incubator.

Flask	Shelf	Lower limit from Step 8.10.83 B (DDMMMYY HHMM)	Upper limit from Step 8.10.83 C (DDMMMYY HHMM)	Time of Removal from Incubator (DDMMMYY HHMM)	Is 8.10.83 B < Time of Removal from Incubator < Step 8.10.83 C Yes/No*

NOTE: This step must was performed as each flask is removed from the incubator.

8.12.3 Prepared TIL collection bag Labeled a 3000mL collection bag as "TIL Suspension", lot number, and initial/date.

8.12.4 Sealed off extra connections. Heat sealed off two leur connections on the collection bag near the end of each connection per Process Note 5.12.

8.12.5 GatheRex Setup. Sterile welded (per Process Note 5.11) the red media removal line from the G-Rex500MCS to the 10L labtainer bag prepared in Step 8.11.5. NOTE: Referenced Process Note 5.16 for use of multiple GatheRex devices.

8.12.6 GatheRex Setup. Sterile welded (per Process Note 5.11) the clear cell removal line from the G-Rex500MCS to the TIL Suspension collection bag prepared in Step 8.12.3. NOTE: Reference Process Note 5.16 for use of multiple GatheRex devices.

8.12.7 GatheRex Setup. Placed the G-Rex500MCS flask on the left side of the GatheRex. Placed the supernatant Labtainer bag and pooled TIL suspension collection bag to the right side.

8.12.8 GatheRex Setup. Installed the red media removal line from the G-Rex500MCS to the top clamp (marked with a red line) and tubing guides on the GatheRex. Installed the clear harvest line from the G-Rex500MCS to the bottom clamp (marked with a blue line) and tubing guides on the GatheRex.

8.12.9 GatheRex Setup. Attached the gas line from the GatheRex to the sterile filter of the G-Rex500MCS. NOTE: Before removing the supernatant from the G-Rex500MCS, ensured all clamps on the cell removal lines were closed.

8.12.10 Volume Reduction. Transferred ~4.5L of supernatant from the G-Rex500MCS to the Supernatant bag. Visually inspected G-Rex500MCS to ensure flask is level and media had been reduced to the end of the aspirating dip tube. Repeat step if needed. NOTE: If the GatheRex stopped prematurely, it may be restarted by pressing the button with the arrow pointing to the right again.

8.12.11 Prepared flask for TIL Harvest. After removal of the supernatant, closed all clamps to the red line.

8.12.12 Initiated collection of TIL. Recorded the start time of the TIL harvest.

8.12.13 Initiated collection of TIL. Vigorously tap flask and swirl media to release cells. Performed an inspection of the flask to ensure all cells have detached. Placed "TIL Suspension" 3000mL collection bag on dry wipes on a flat surface. NOTE: Contacted area management if cells did not detach.
8.12.14 Initiated collection of TIL. Tilted the flask to ensure hose is at the edge of the flask. NOTE: If the cell collection hose was not at the junction of the wall and bottom membrane, rapping the flask while tilted at a 45° angle is usually sufficient to properly position the hose.
8.12.15 TIL Harvest. Released all clamps leading to the TIL suspension collection bag.
8.12.16 TIL Harvest. Using the GatheRex, transferred the TIL suspension into the 3000mL collection bag. NOTE: Maintained the tilted edge until all cells and media were collected.
8.12.17 TIL Harvest. Inspect membrane for adherent cells.
8.12.18 Rinsed flask membrane. Rinsed the bottom of the G-Rex500MCS. Covered ~1/4 of gas exchange membrane with rinse media.
8.12.19 Close clamps on G- Rex500MCS. Ensure all clamps are closed.
8.12.20 Heat sealed. Heat seal (per Process Note 5.12) the collection bag containing the TIL as close to the weld as possible so that the overall tubing length remained approximately the same.
8.12.21 Heat Sealed. Heat sealed (per Process Note 5.12) the Supernatant bag.
8.12.22 Completed harvest of remaining G-Rex 500 MCS flasks. Repeat Steps 8.12.2 and 8.12.5 - 8.12.21, pooling all TIL into the same collection bag.
NOTE: IT WAS NECESSARY TO REPLACE THE 10L SUPERNATANT BAG AFTER EVERY 2ND FLASK. NOTE: Reference Process Note 5.16 for use of multiple GatheRex devices.
8.12.23 Prepared LOVO source bag. Obtained a new 3000mL collection bag. Labeled as "LOVO Source Bag", lot number, and Initial/Date.
8.12.24 Prepared LOVO source bag. Heat sealed (per Process Note 5.12) the tubing on the "LOVO Source bag", removing the female luers, leaving enough line to weld.
8.12.25 Weighed LOVO Source Bag. Placed an appropriately sized plastic bin on the scale and tare. Place the LOVO Source Bag, including ports and lines, in the bin and record the dry weight
8.12.26 Transferred cell suspension into LOVO source bag. Closed all clamps of a 170 µm gravity blood filter.
8.12.27 Transferred cell suspension into LOVO source bag. Sterile welded (per Process Note 5.11) the long terminal end of the gravity blood filter to the LOVO source bag.
8.12.28 Transferred cell suspension into LOVO source bag. Sterile welded (per Process Note 5.11) one of the two source lines of the filter to "pooled TIL suspension" collection bag.
8.12.29 Transferred cell suspension into LOVO source bag. Once weld was complete, heat sealed (per Process Note 5.12) the unused line on the filter to remove it.
8.12.30 Transferred cell suspension into LOVO source bag. Opened all necessary clamps and elevate the TIL suspension by hanging the collection

bag on an IV pole to initiate gravity-flow transfer of TIL through the blood filter and into the LOVO source bag. Gently rotated or knead the TIL Suspension bag while draining in order to keep the TIL in even suspension. Note: Did not allow the LOVO source bag to hang from the filtration apparatus. Laid LOVO source bag on dry wipes on a flat surface.

8.12.31 Closed all clamps. Once all TIL were transferred to the LOVO source bag, closed all clamps.

8.12.32 Heat Sealed. Heat sealed (per Process Note 5.12) as close to weld as possible to remove gravity blood filter.

8.12.33 Removed Cell Counts Samples. In the BSC, using separate 3mL syringes for each sample, removed 4x1.0mL cell count samples from the LOVO source bag using the needless injection port. Placed samples in the cryovials prepared in Step 8.11.36. NOTE: Wiped needless injection port with an alcohol pad and mix LOVO source bag between each sample.

8.12.34 Performed Cell Counts. Performed cell counts and calculations utilizing NC-200 and Process Note 5.14. Diluted cell count samples initially by adding 0.5mL of cell suspension into 4.5mL of AIM-V media prepared in Step 8.11.35. This gave a 1:10 dilution.

8.12.35 Recorded Cell Count sample volumes.

8.12.36 Determined Multiplication Factor

Parameter	Formula	Result
Total cell count sample Volume	8.12.35A + 8.12.35B	C. µL
Multiplication Factor	C ÷ 8.12.35A	D.

8.12.37 Selected protocols and enter multiplication factors. Ensured the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0

8.12.38 Record Cell Counts from Nucleoview

8.12.39 Determined the Average Viability, Viable Cell Concentration, and Total Nucleated Cell concentration of the cell counts performed.

Parameter	Formula	Result
Viability	(8.12.38A + 8.12.38B) ÷ 2	G.
Viable Cell Concentration	(8.12.38C + 8.12.38D) ÷ 2	H. cells/mL
Total Nucleated Cell Concentration	(8.12.38E + 8.12.38F) ÷ 2	I. cells/mL

8.12.40 Determined Upper and Lower Limit for counts

Parameter	Formula	Result
Lower Limit	8.12.39H x 0.9	J. cells/mL
Upper Limit	8.12.39I x 1.1	K. cells/mL

8.12.41 Were both counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	8.12.38 C and D ≥ 8.12.40J	
Upper Limit	8.12.38 C and D ≤ 8.12.40K	

NOTE: If either result was "No" performed second set of counts in steps 8.12.42 – 8.12.49.

8.12.42 If Applicable: Performed cell counts. Performed cell counts and calculations in utilizing NC-200 and Process Note 5.14. NOTE: Dilution may be adjusted according based off the expected concentration of cells.

8.12.43 If Applicable: Recorded Cell Count sample volumes

8.12.44 If Applicable: Determined Multiplication Factor

Parameter	Formula	Result
Total cell count sample Volume	8.12.43A + 8.12.43B	C. µL
Multiplication Factor	C ÷ 8.12.43A	D.

8.12.45 If Applicable: Selected protocols and enter multiplication factors.Ensure the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered. NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0

8.12.46 If Applicable: Record Cell Counts from Nucleoview

8.12.47 If Applicable: Determine the Average Viability, Viable Cell Concentration, and Total Nucleated Cell concentration of the cell counts performed.

Parameter	Formula	Result
Viability	(8.12.46A + 8.12.46B) ÷ 2	G.
Viable Cell Concentration	(8.12.46C + 8.12.46D) ÷ 2	H. cells/mL
Total Nucleated Cell Concentration	(8.12.46E + 8.12.46F) ÷ 2	I. cells/mL

8.12.48 If Applicable: Determined Upper and Lower Limit for counts

Parameter	Formula	Result
Lower Limit	8.12.47 H x 0.9	J. cells/mL
Upper Limit	8.12.47 H x 1.1	K. cells/mL

8.12.49 If Applicable: Were counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	8.12.46 C and D ≥ 8.12.48J	
Upper Limit	8.12.46 C and D ≤ 8.12.48K	

NOTE: If either result was "No" continue to Step 8.12.50 to determine an average

8.12.50 If Applicable: Determined an average Viable Cell Concentration and average Total Nucleated Cell Concentration from all four counts performed.

8.12.51 QA Review of Cell Counts. QA personnel review calculations performed in steps 8.12.38 – 8.12.50.

8.12.52 Weighed LOVO Source Bag. Placed an appropriately sized plastic bin on the scale and tare. Placed the full LOVO source bag in the bin and record the weight. Calculated the volume of cell suspension.

8.12.53 Calculate Total Viable TIL Cells.

Parameter	Formula	Result
Average Viable Cell Concentraion*	8.12.39 H* -or- 8.12.47 H* -or- 8.12.50 E*	A. cells/mL
Total Volume	8.12.52 C	B. mL
Total Viable Cells	A x B	C. cells
Is C ≥ $1.5x10^9$?	Yes/No**	

*Circled step reference used to determine Viable Cell Concentration.

**If "Yes," proceeded. If "No," contacted Area Management.

8.12.54 Calculate Total Nucleated Cells.

Parameter	Formula	Result
Average Total Nucleated Cell Concentraion*	8.12.39 I* -or- 8.12.47 I* -or- 8.12.50 J*	A. cells/mL
Total Volume	8.5.52 C	B. mL
Total Nucleated Cells	A x B	C. cells

*Circled step reference used to determine Total Nucleated Cell Concentration.

8.12.55 Prepared Mycoplasma Diluent. In the BSC, removed 10.0 mL from one supernatant bag via luer sample port and placed in a 15mL conical. Label 15mL conical "Mycoplasma Diluent" and keep in the BSC for use in Step 8.14.69.

8.12.56 Review Section 8.12

8.13 LOVO

8.13.1 Turned on the LOVO using the switch on the back left of the instrument. NOTE: Steps 8.13.1-8.13.13 may be performed concurrently with Sections 8.11-8.12.

8.13.2 Checked weigh scales and pressure sensor.

8.13.3 Made sure there was nothing hanging on any of the weigh scales and reviewed the reading for each scale. Recorded values in Step 8.13.5. Note: If any of the scales read outside of a range of 0 +/- 2 g, performed weigh scale calibration

8.13.4 If all scales were in tolerance with no weight hanging, proceeded to hang a 1- kg weight on each scale (#1-4) and reviewed the reading. Recorded Values in Step 8.13.5.

8.13.5 Scale Checked. Recorded the displayed values for each scale. If values were in range, continue processing. If values were not in range, perform Calibration.
8.13.6 Reviewed the pressure sensor reading on the Instrument Operation Profile Screen and recorded. The acceptable range for the pressure reading was 0 +/- 10 mmHg. If displayed value was out of this range, stored a new atmospheric pressure setting, per the machine instructions.
8.13.7 Repeated steps. If a new weigh scale calibration had been performed or a new atmospheric pressure setting had been stored, repeated Steps 8.13.3 – 8.13.6.
8.13.8 Started the "TIL G-Rex Harvest" protocol from the drop-down menu.
8.13.9 The Solution 1 Screen displayed: Buffer type read PlasmaLyte
8.13.10-8.13.16 Followed the LOVO touch screen prompts.
8.13.17 Determined the final product target volume.
NOTE: Using the total nucleated cells (TNC) value from Step 8.12.54 C and the chart below, determined the final product target volume. Recorded the Final Product Volume (mL)

Cell Range	Final Product (Retentate) Volume to Target (mL)
0 < Total (Viable + Dead) Cells $\leq 7.1 X10^{10}$	165
$7.1X10^{10}$ < Total (Viable + Dead) Cells $\leq 1.1X10^{11}$	215
$1.1X10^{11}$ < Total (Viable + Dead) Cells $\leq 1.5X10^{11}$	265

Note: If TVC from Step 8.12.53 C was $>1.5x10^{11}$ contacted Area Management.
8.13.18 – 8.13.22 Followed the LOVO touch screen prompts.
8.13.23 Loaded disposable kit. Prior to loading the disposable kit, wipe pressure sensor port with an alcohol wipe followed by a lint-free wipe. Load the disposable kit. Follow screen directions on loading the disposable kit.
8.13.24 Removed filtrate bag. When the standard LOVO disposable kit had been loaded, touched the Next button. The Container Information and Location Screen displayed. Removed filtrate bag from scale
8.13.25 Ensured Filtrate container was New and Off-Scale
8.13.26 Entered Filtrate capacity. Sterile welded a LOVO Ancillary Bag onto the male luer line of the existing Filtrate Bag. Ensured all clamps are open and fluid path is clear. Touch the Filtrate Container Capacity entry field. A numeric keypad displays. Enter the total new Filtrate capacity (5,000mL). Touch the button to accept the entry. NOTE: Estimated Filtrate Volume should not exceed 5000 mL.
8.13.27 Placed Filtrate container on benchtop. NOTE: If tubing was removed from the F clamp during welding, placed the tubing back into the clamp. Placed the new Filtrate container on the benchtop. DID NOT hang the Filtrate bag on weigh scale #3. Weigh scale #3 will be empty during the procedure.
8.13.28 Followed the LOVO touch screen prompts after changes to the filtrate container.

8.13.29 Ensured kit was loaded properly. The Disposable Kit Dry Checks overlay displays. Checked that the kit was loaded properly and all clamps were open. Checked all tubing for kinks or other obstructions and correct if possible. Ensured kit was properly installed and check all Robert's clamps. Pressed the Yes button. All LOVO mechanical clamps closed automatically and the Checking Disposable Kit Installation screen displays. The LOVO went through a series of pressurizing steps to check the kit.
8.13.30 Kit Check Results. If the Kit check passed, proceeded to the next step. *If No, a second Kit Check could be performed after checks have been complete. *If No, Checked all tubing for kinks or other obstructions and correct *If No, Ensured kit was properly installed and check all Robert's clamps. If the 2nd kit check failed: Contact area management and prepare to installation of new kit in Section 10.0. Repeat Step 8.13.23-Step 8.13.30 needed.
8.13.31 Attached PlasmaLyte. The Connect Solutions screen displayed. The wash value would always be 3000 mL. Entered this value on screen. Sterile welded the 3000mL bag of PlasmaLyte to the tubing passing through Clamp 1 per Process Note 5.11. Hung the PlasmaLyte bag on an IV pole placing both corner bag loops on the hook.
8.13.32 Verified that the PlasmaLyte was attached. Opened any plastic clamps. Verified that the Solution Volume entry was 3000mL. Touched the "Next" button. The Disposable Kit Prime overlay displayed. Verified that the PlasmaLyte was attached and any welds and plastic clamps on the tubing leading to the PlasmaLyte bag were open, then touched the Yes button
8.13.33 Observed that the PlasmaLyte is moving. Disposable kit prime starts and the Priming Disposable Kit Screen displays. Visually observed that PlasmaLyte moving through the tubing connected to the bag of PlasmaLyte. If no fluid was moving, pressed the Pause Button on the screen and determined if a clamp or weld was still closed. Once the problem had been solved, pressed the Resume button on the screen to resume the Disposable Kit Prime. When disposable kit prime finished successfully, the Connect Source Screen displayed.
8.13.34-8.13.35 Followed the LOVO touch screen prompts.
8.13.36 Attached Source container to tubing. Sterile weld the LOVO Source Bag prepared in Step 8.12.31 to the tubing passing through Clamp S per Process Note 5.11. It could be necessary to remove the tubing from the clamp. Note: Made sure to replace source tubing into the S clamp if removed.
8.13.37 Hung Source container. Hung the Source container on the IV pole placing both corner bag loops on the hook. DID NOT hang the Source on weigh scale #1. Opened all clamps to the source bag.
8.13.38 Verified Source container was attached. Touched the Next button. The Source Prime overlay displayed. Verified that the Source was attached to the disposable kit, and that any welds and plastic clamps on the tubing leading to the Source were open. Touched the Yes button.
8.13.39 Confirm PlasmaLyte was moving. Source prime started and the Priming Source Screen displayed. Visually observed that PlasmaLyte is moving through the tubing attached to the Source bag. If no fluid is moving, press the Pause Button on the screen and determine if a clamp or weld is still closed. Once the problem was solved, pressed the Resume button on the screen to resume the Source Prime.

8.13.40 Started Procedure Screen. When the Source prime finishes successfully, the Start Procedure Screen displays. Pressed Start, the "Pre-Wash Cycle 1" pause screen appears immediately after pressing start.
8.13.41 Inverted In Process Bag. Removed the In Process Bag from weigh scale #2 (can also remove tubing from the In Process top port tubing guide) and manually invert it to allow the wash buffer added during the disposable kit prime step to coat all interior surfaces of the bag. Re-hang the In Process Bag on weigh scale #2 (label on the bag was facing to the left). Replace the top port tubing in the tubing guide, if it was removed.
8.13.42 Inverted Source bag. Before pressing the Start button, mixed the Source bag without removing it from the IV pole by massaging the bag corners and gently agitating the cells to create a homogeneous cell suspension. Pressed the Resume button. The LOVO started processing fluid from the Source bag and the Wash Cycle 1 Screen displays.
8.13.43 Source Rinse Pause. The Rinse Source Pause screen displayed once the source container is drained and the LOVO had added wash buffer to the Source bag. Without removing the Source bag from IV pole, massaged the corners and mixed well. Pressed Resume.
8.13.44 Mix In Process Bag Pause. To prepare cells for another pass through the spinner, the In Process Bag was diluted with wash buffer. After adding the wash buffer to the In Process Bag, the LOVO pauses automatically and displays the "Mix In Process Bag" Pause Screen. Without removing the bag from the weigh scale, mixed the product well by gently squeezing the bag. Press Resume.
8.13.45 Massage In Process Corners Pause. When the In Process Bag was empty, wash buffer was added to the bottom port of the In Process Bag to rinse the bag. After adding the rinse fluid, the LOVO paused automatically and displayed the "Massage IP corners" Pause Screen. When the "Massage IP corners" Pause Screen displayed, DO NOT remove the bag from weigh scale #2. With the In Process Bag still hanging on weigh scale #2, massage the corners of the bag to bring any residual cells into suspension. Ensured the bag was not swinging on the weigh scale and pressed the Resume button.
8.13.46 Wait for Remove Products Screen. At the end of the LOVO procedure, the Remove Products Screen displayed. When this Screen displays, all bags on the LOVO kit could be manipulated. Note: Did not touch any bags until the Remove Products displayed.
8.13.47 Removed retentate bag. Placed a hemostat on the tubing very close to the port on the Retentate bag to keep the cell suspension from settling into the tubing. Heat sealed (per Process Note 5.12) below the hemostat, making sure to maintain enough line to weld in Step 8.13.48. Removed the retentate bag.
8.13.48 Prepared retentate bag for formulation. Welded (per Process Note 5.11) the female luer lock end of a 4" Plasma Transfer Set to the retentate bag. Transferred the retentate bag to the BSC for use in Step 8.14.11.
8.13.49 Removed Products. Followed the instructions on the Remove Products Screen. Closed all clamps on the LOVO kit to prevent fluid movement.
8.13.50 Removed Products. Touched the Next button. All LOVO mechanical clamps opened and the Remove Kit Screen displayed.

8.13.51 Recorded Data. Followed the instructions on the Remove Kit screen. Touched the "Next" button. All LOVO mechanical clamps close and the Results Summary Screen displays. Recorded the data from the results summary screen in table exactly as they are displayed. Closed all pumps and filter support. Removed the kit when prompted to do so by the LOVO. *NOTE: All Times recorded were recorded directly from the LOVO Results Summary Screen in HH:MM:SS format and (HH:MM:SS) format when applicable

8.13.52-8.13.54 Protocol Selection through LOVO shutdown. Follow the LOVO screen prompts.

8.13.55 Review Section 8.13

8.14 Final Formulation and Fill

8.14.1 Target volume/bag calculation. From the table below, selected the number of CS750 bags to be filled, target fill volume per bag, volume removed for retain per bag, and final target volume per bag that corresponded to the Volume of LOVO Retentate from Step 8.13.22.

Volume of LOVO product	Volume of CS10 to add to product	Final Predicted Volume of formulated product	Number of bags to be filled	Target Fill Volume per bag	Volume removed for retain per bag	Final Target Volume per bag
165mL	165mL	330mL	3	107mL	7mL	100mL
215mL	215mL	430mL	4	105mL	5mL	100mL
265mL	265mL	530mL	4	130mL	5mL	125mL

8.14.2 Prepared CRF Blank. Calculated volume of CS-10 and LOVO wash buffer to formulate blank bag.

Final Target Volume per Bag 8.14.1E A	Blank LOVO Wash Buffer Volume B = A / 2	Blank CS-10 Volume (mL) C = B
mL	mL	mL

8.14.3 Prepared CRF Blank. Outside of the BSC, using the syringe of LOVO Wash Buffer prepared in Step 8.11.29, added volume calculated in Step 8.14.2 B to an empty CS750 bag via luer connection. Note: Blank CS750 bag formulation does not need to be done aseptically.

8.14.4 Prepared CRF Blank Using an appropriately sized syringe, added the volume of CS-10 calculated in Step 8.14.2 to the same CS750 bag prepared in Step 8.14.3. Placed a red cap on the CS750 bag.

8.14.5 Prepared CRF Blank. Removed as much air as possible from the CS-750 bag as possible. Heat sealed (per Process Note 5.12) the CS750 bag as close to the bag as possible, removing the tubing.

8.14.6 Prepared CRF Blank.Label CS750 bag with "CRF Blank", lot number, and initial/date. Placed the CRF Blank on cold packs until it was placed in the CRF.

8.14.7 Calculated required volume of IL-2. Calculated the volume of IL-2 to add to the Final Product

Parameter	Formula	Result
Final Retentate Volume	Step 8.13.51	A. mL
Final Formulated Volume	B = A x 2	B. mL
Final IL-2 Concentration desired (IU/mL)	300 IU/mL	C. 300 IU/mL
IU of IL-2 Required	D = B x C	D. IU
IL-2 Working Stock from Step 8.11.33	6 x 10^4 IU/mL	E. 6 x 10^4 IU/mL
Volume of IL-2 to Add to Final Product	F = D ÷ E	F. mL

8.14.8 Assembled Connect apparatus. Sterile welded (per Process Note 5.11) a 4S-4M60 to a CC2 Cell Connect replacing a single spike of the Cell Connect apparatus (B) with the 4-spike end of the 4S-4M60 manifold at (G).
8.14.9 Assembled Connected apparatus. Sterile welded (per Process Note 5.11) the CS750 Cryobags to the harness prepared in Step 8.14.8, replacing one of the four male luer ends (E) with each bag. Reference Step 8.14.1 to determine the number of bags needed.
8.14.10 Assembled Connected apparatus. Welded (per Process Note 5.11) CS-10 bags to spikes of the 4S-4M60. Kept CS-10 cold by placing the bags between two cold packs conditioned at 2-8°C.
8.14.11 Passed materials into the BSC.

Item	Item # or Step Reference	Quantity
4" plasma transfer set		1
IL-2 (6.0x10^4) aliquot	8.11.33	1
Appropriate size syringe to add IL2	8.14.7F	1
LOVO retentate bag	8.13.48	1
Red Caps		5

8.14.12 Prepared TIL with IL-2. Using an appropriately sized syringe, removed amount of IL-2 determined in Step 8.14.7 from the "IL-2 6x10^4" aliquot.
8.14.13 Prepared TIL with IL-2. Connect the syringe to the retentate bag prepared in Step 8.13.48 via the Luer connection and inject IL-2.
8.14.14 Prepare TIL with IL-2Clear the line by pushing air from the syringe through the line.
8.14.15 Labeled Formulated TIL Bag. Closed the clamp on the transfer set and label bag as "Formulated TIL" and passed the bag out of the BSC.
8.14.16 If applicable: Sample per sample plan. If there was remaining "IL-2 6x10^4" aliquot prepared in step 8.11.33, remove a ~5 mL sample retain according to the sample plan using an appropriately sized syringe and dispense into a 50 mL conical tube.
8.14.17 If applicable: Sampled per sample plan. Labeled with sample plan inventory label and stored at 2-8ºC until submitted to Login for testing per Sample Plan.

8.14.18 If applicable: Sampled per sample plan. Ensured that LIMS sample plan sheet was filled out for removal of the sample.
8.14.19 Added the Formulated TIL bag to the apparatus. Once IL-2 had been added, welded (per Process Note 5.11) the "Formulated TIL" bag to the remaining spike (A) on the apparatus prepared in Step 8.14.10.
8.14.20 Added CS10. Passed the assembled apparatus with attached Formulated TIL, CS-750 bags, and CS-10 into the BSC. NOTE: The CS-10 bag and all CS-750 bags were placed between two cold packs preconditioned at 2-8°C. Did not place Formulated TIL bag on cold packs.
8.14.21 Added CS10. Ensured all clamps were closed on the apparatus. Turn the stopcock so the syringe was closed.
8.14.22 Switched Syringes. Drew ~10mL of air into a 100mL syringe and replaced the 60mL syringe on the apparatus.
8.14.23 Added CS10. Turned stopcock so that the line to the CS750 bags is closed. Open clamps to the CS-10 bags and pull volume calculated in Step 8.14.1B into syringe. NOTE: Multiple syringes will be used to add appropriate volume of CS-10. NOTE: Record volume from each syringe in Step 8.14.26
8.14.24 Added CS10. Closed clamps to CS-10 and open clamps to the Formulated TIL bag and add the CS-10. Note: Add first 10.0mL of CS10 at approximately 10.0mL/minute. Add remaining CS-10 at approximate rate of 1.0mL /sec. Note: Multiple syringes were used to add appropriate volume of CS-10. Did not reuse a syringe once it had been dispensed.
8.14.25 Added CS10. Recorded time. NOTE: The target time from first addition of CS-10 to beginning of freeze is 30
8.14.26 Added CS10. Recorded the volume of each CS10 addition and the total volume added. Total volume match calculated volume from Step 8.14.1B
8.14.27 Added CS10. Closed all clamps to the CS10 bags.
8.14.28 Prepared CS-750 bags. Turned the stopcock so that the syringe was open. Opened clamps to the Formulated TIL bag and drew up suspension stopping just before the suspension reaches the stopcock.
8.14.29 Prepared CS-750 bags. Closed clamps to the formulated TIL bag. Turned stopcock so that it was open to the empty CS750 final product bags.
8.14.30 Prepared CS-750 bags. Using a new syringe, removed as much air as possible from the CS750 final product bags by drawing the air out. While maintaining pressure on the syringe plunger, clamped the bags shut.
8.14.31 Prepared CS-750 bags. Draw ~20mL air into a new 100mL syringe and connect to the apparatus.
NOTE: Each CS-750 final product bag should be between two cold packs to keep formulated TIL suspension cold.
8.14.32 Dispensed cells. Turned the stopcock so the line to the final product bags was closed. Pulled the volume calculated in Step 8.14.1 from the Formulated TIL bag into the syringe. NOTE: Multiple syringes could be used to obtain correct volume.
8.14.33 Dispensed cells. Turned the stopcock so the line to the formulated TIL bag is closed. Working with one final product bag at a time, dispense cells into a final product bag. Recorded volume of cells added to each CS750 bag in Step 8.14.35

8.14.34 Dispensed cells. Cleared the line with air from the syringe so that the cells are even with the top of the spike port. Closed the clamp on the filled bag. Repeated Step 8.14.29- Step 8.14.34 for each final product bag, gently mixing formulated TIL bag between each.
8.14.35 Dispensed cells. Record volume of TIL placed in each final product bag below.
8.14.36 Removed air from final product bags and take retain. Once the last final product bag was filled, closed all clamps.
8.14.37 Removed air from final product bags and take retain. Drew 10mL of air into a new 100mL syringe and replace the syringe on the apparatus.
8.14.38 Removed air from final product bags and take retain. Manipulating a single bag at a time, drew all of the air from each product bag plus the volume of product for retain determined in Step 8.14.1 D. NOTE: Upon removal of sample volume, inverted the syringe and used air to clear the line to the top port of the product bag. Clamped the line to the bag once the retain volume and air was removed.
8.14.39 Recorded Volume Removed. Recorded volume of retain removed from each bag.
8.14.40 Dispensed Retain. Dispensed retain into a 50mL conical tube and label tube as "Retain" and lot number. Repeat Step 8.14.37- Step 8.14.39 for each bag.
8.14.41 Prepared final product for cryopreservation. With a hemostat, clamped the lines close to the bags. Removed syringe and red cap luer connection on the apparatus that the syringe was on. Passed apparatus out of the BSC.
8.14.42 Prepared final product for cryopreservation. Heat sealed (per Process Note 5.12) at F, removing the empty retentate bag and the CS-10 bags. NOTE: Retained luer connection for syringe on the apparatus. Disposed of empty retentate and CS-10 Bags.
8.14.43 Performed visual inspection. NOTE: Step 8.14.43 – Step 8.14.46 may be performed concurrently with Step 8.14.47- Step 8.14.68.
8.14.44 Final Product Label Sample. Labeled final product bags. Attached sample final product label below.
8.14.45 Prepared final product for cryopreservation. Held the cryobags on cold pack or at 2-8°C until cryopreservation.
8.14.46 Prepared external labels. Ensured the QA issued external labels that will be attached to the cassettes labels match corresponding final product label. Attached QA issued external labels to cassettes. Attached a sample external label below:
8.14.47 Removed Cell Count Sample. Using an appropriately sized pipette, remove 2.0 mL of retain removed in Step 8.14.38 and place in a 15mL conical tube to be used for cell counts.
8.14.48 Performed Cell Counts. Performed cell counts and calculations utilizing the NC-200 per SOP-00314 and Process Note 5.14. NOTE: Diluted only one sample to appropriate dilution to verify dilution is sufficient. Diluted additional samples to appropriate dilution factor and proceed with counts.
8.14.49 Recorded Cell Count sample volumes. NOTE: If no dilution needed, "Sample [µL]" = 200, "Dilution [µL]" =0
8.14.50 Determined Multiplication Factor

Parameter	Formula	Result
Total cell count sample Volume	8.14.49A + 8.14.49B	C. µL
Multiplication Factor	C ÷ 8.14.49A	D.

8.14.51 Select protocols and enter multiplication factors. Ensure the "Viable Cell Count Assay" protocol has been selected, all multiplication factors, and sample and diluent volumes have been entered per SOP – 00314
NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0
8.14.52 Recorded File Name, Viability and Cell Counts from Nucleoview.
8.14.53 Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	(8.14.52A + 8.14.52B) ÷ 2	E. %
Viable Cell Concentration	(8.14.52C + 8.14.52D) ÷ 2	F. cells/mL

8.14.54 Determined Upper and Lower Limit for counts.

Parameter	Formula	Result
Lower Limit	8.14.53F x 0.9	G. cells/mL
Upper Limit	8.14.53F x 1.1	H. cells/mL

8.14.55 Were both counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	8.14.52 C and D ≥ 8.14.54G	
Upper Limit	8.14.52 C and D ≤ 8.14.54H	

NOTE: If either result is "No" perform second set of counts in steps 8.14.56 – 8.14.63
8.14.56 If Applicable: Performed cell counts. Performed cell counts and calculations in utilizing NC-200 per SOP-00314 and Process Note 5.14.
NOTE: Dilution may be adjusted according based off the expected concentration of cells.
8.14.57 If Applicable: Recorded Cell Count sample volumes.
8.14.58 If Applicable: Determined Multiplication Factor

Parameter	Formula	Result
Total cell count sample Volume	8.14.57A + 8.14.57B	C. mL
Multiplication Factor	C ÷ 8.14.57A	D.

8.14.59 If Applicable: Selected protocols and entered multiplication factors. Ensured the "Viable Cell Count Assay" protocol had been selected, all multiplication factors, and sample and diluent volumes had been entered.
NOTE: If no dilution needed, enter "Sample [µL]" = 200, "Dilution [µL]" = 0
8.14.60 If Applicable: Recorded Cell Counts from Nucleoview

8.14.61 If Applicable: Determined the Average of Viable Cell Concentration and Viability of the cell counts performed.

Parameter	Formula	Result
Viability	(8.14.60A + 8.14.60B) ÷ 2	E. %
Viable Cell Concentration	(8.14.60C + 8.14.60D) ÷ 2	F. cells/mL

8.14.62 If Applicable: Determined Upper and Lower Limit for counts.

Parameter	Formula	Result
Lower Limit	8.14.61F x 0.9	G. cells/mL
Upper Limit	8.14.61F x 1.1	H. cells/mL

8.14.63 If Applicable: Were counts within acceptable limits?

Parameter	Formula	Result (Yes/No)
Lower Limit	8.14.60 C and D ≥ 8.14.62G	
Upper Limit	8.14.60 C and D ≤ 8.14.62H	

NOTE: If either result is "No" continue to Step 8.14.64 to determine an average.

8.14.64 If Applicable: Determined an average Viable Cell Concentration from all four counts performed.

8.14.65 Calculated Flow. Cytometry Sample. Performed calculation to ensure sufficient cell concentration for flow cytometry sampling.

Viable Cell Concentration From Step 8.14.53 F* Or Step 8.14.61 F* Or Step 8.14.64 E* A	Target Volume Required for $6x10^7$ TVC B = $6x10^7$ cells/ A	Is B ≤ 1.0 mL? (Yes/No**)
cells/mL	mL	

*Circle step reference used to determine Viable Cell Concentration **NOTE: If "No", contact area management.

8.14.66 Calculated IFN-γ. Sample Performed calculation to ensure sufficient cell concentration for IFN-γ sampling.

Viable Cell Concentration From Step 8.14.53 F* Or Step 8.14.61 F* Or Step 8.14.64 E* A	Volume Required for Minimum $1.5x10^7$ TVC B = $1.5x10^7$ cells / A	Is B ≤ 1.0 mL? (Yes/No**)
cells/mL	mL	

*Circle step reference used to determine Viable Cell Concentration **NOTE: If "No", contact area management.

8.14.67 Reported Results. Completed forms for submission with samples.

8.14.68 Heat Sealed. Once sample volumes had been determined, heat sealed (per Process Note 5.12) Final Product Bags as close to the bags as possible to remove from the apparatus.

8.14.69 Labeled and Collected Samples per Sample Plan.

Sample	Number of Containers	Sample Volume to Add to Each	Container Type	Destination
*Mycoplasma	1	1.0 mL	15 mL Conical	Login
Endotoxin	2	1.0 mL	2 mL Cryovial	Login
Gram Stain	1	1.0 mL	2 mL Cryovial	Login
IFN-g	1	1.0 mL	2 mL Cryovial	Login
Flow Cytometry	1	1.0 mL	2 mL Cryovial	Login
**Bac-T Sterility	2	1.0 mL	Bac-T Bottle	Login
QC Retain	4	1.0 mL	2 mL Cryovial	CRF
Satellite Vials	10	0.5 mL	2 mL Cryovial	CRF

*NOTE: For the Mycoplasma sample, add formulated cell suspension volume to the 15mL conical labelled "Mycoplasma Diluent" from Step 8.12.55.

**NOTE: Proceed to Step 8.14.70 for Bac-T inoculation.

8.14.70 Sterility & BacT. Testing Sampling. In the BSC, remove a 1.0mL sample from the retained cell suspension collected in Step 8.14.38 using an appropriately sized syringe and inoculate the anaerobic bottle. Repeat the above for the aerobic bottle. NOTE: Store Bac-T bottles are room temperature and protect from light.

8.14.71 Labeled and stored samples. Labeled all samples with sample plan inventory labels and store appropriately until transfer to Login. NOTE: Proceeded to Section 8.15 for cryopreservation of final product and samples.

8.14.72 Signed for sampling. Ensured that LIMS sample plan sheet is completed for removal of the samples.

8.14.73 Sample Submission. Submitted all Day 22 testing samples to Login.

8.14.74 Environmental Monitoring. After processing, verified BSC and personnel monitoring had been performed.

8.14.75 Review Section 8.14

8.15 Final Product Cryopreservation

8.15.1 Prepared Controlled Rate Freezer. Verified the CRF had been set up prior to freeze. Record CRF Equipment. Cryopreservation is performed.

8.15.2 Set up CRF probes. Punctured the septum on the CRF blank bag. Inserted the 6mL vial temperature probe.

8.15.3 Placed final product and samples in CRF. Placed blank bag into preconditioned cassette and transferred into the approximate middle of the CRF rack. Transferred final product cassettes into CRF rack and vials into CRF vial rack.

8.15.4 Placed final product and samples in CRF. Transferred product racks and vial racks into the CRF. Recorded the time that the product is transferred

into the CRF and the chamber temperature in Step 8.15.5. NOTE: Evenly distributed the cassettes and vial rack in the CRF, allowed as much space as possible between each shelf.

8.15.5 Determined the time needed to reach 4 ºC ± 1.5 ºC and proceed with the CRF run. Once the chamber temperature reached 4 ºC ± 1.5 ºC, started the run. Recorded time.

Parameter	Formula	Value
Time Final Product is transferred to CRF	(HHMM)	
Temperature Final Product is transferred into CRF	From monitor	ºC
B. CRF Start Time	(HHMM)	
Elapsed Time from Formulation to CRF Start	C = B – Step 8.14.25A	min

8.15.6 CRF Completed and Stored. Stopped the CRF after the completion of the run. Remove cassettes and vials from CRF. Transferred cassettes and vials to vapor phase LN2 for storage. Recorded storage location

POST PROCESSING SUMMARY

- Post-Processing: Final Drug Product
  - (Day 22) Determination of CD3+ Cells on Day 22 REP by Flow Cytometry
  - (Day 22) Gram Staining Method (GMP)
  - (Day 22) Bacterial Endotoxin Test by Gel Clot LAL Assay (GMP)
  - (Day 16) BacT Sterility Assay (GMP)
  - (Day 16) Mycoplasma DNA Detection by TD-PCR (GMP)
  - Acceptable Appearance Attributes (Step 8.14.43)
  - (Day 22) BacT Sterility Assay (GMP)
  - (Day 22) IFN-gamma Assay

EXAMPLE 2. Genetic Editing of a Cryopreserved TIL Therapy Using TALE Nucleases

[00601] This example describes the use of a genetic editing step in conjunction with a TIL manufacturing process, such as the process shown in Figure 20.

[00602] Optimization of human TIL electroporation is performed as follows. Four-6 pre-REP TIL lines derived from melanoma will be identified that express >25% PD1. Selected TIL lines will be thawed, rested, and activated, working 1 line at a time. Five-million activated TILs will be electroporated with each of the following control or test RNA: no Electroporation control (NE); no RNA control; green fluorescent protein (GFP) mRNA transfection control, CD52 TALEN KO control, PD1 TALEN, LAG-3 TALEN, and TIM-3 TALEN. Viable cells will be counted and an aliquot set aside for transfection efficiency measurements (GFP expression). Electroporated cells will be expanded by REP. Post-REP TILs will be assessed for cell viability and fold expansion (cell counter) and target gene

knockdown (flow and qPCR). The target results are >80% viability or within 10 % of NE; >70% transfection; fold expansion of TILs to within 30% of NE; and >50% knockout.

[00603] Implementation of TALEN-mediated PD-1 knockout to the TIL manufacturing process is performed as follows. Up to 6 fresh tumors from several histologies that may include melanoma, sarcoma, and breast and lung cancer will be used. Research-scale preps will be performed according to the processes shown in Figures 20 and 21. Electroporation conditions from prior experiments will be applied at pre-determined time points of each of the processes. Post-REP TILs will be assessed for: Cell viability and fold expansion (cell counter); Cell phenotypes (flow cytometry); TCR Vβ repertoire (flow); T cell effector functions (IFN-γ production upon re-stimulation); and target gene knockdown (flow and qPCR). The target results are: >80% viability or within 10 % of NE; >70% transfection; fold expansion within 30% of NE; T cell lineages/subsets maintained compared to TILs produced by process 2A; adequate TIL potency; and >50% knockout of PD-1. Following this work, one full-scale preparation of PD-1 knockdown TILs will be carried out and fully characterized.

[00604] Validation of the silencing of two additional target genes in addition to PD-1 will also be tested.

EXAMPLE 3. TALEN-Mediated Inactivation of PD-1 in TILs

[00605] This example describes TALEN-mediated inactivation of PD-1 in conjunction with a TIL manufacturing process described herein, such as a process shown in Figure 20 or Figure 21. The TIL manufacturing process may include early addition of OKT-3 and/or a 4-1BB agonist to the cell culture medium *e.g.,* as illustrated in Embodiment 2 of Figure 21. The OKT-3 and/or 4-1BB agonist may optionally be added beginning on Day 0 or Day 1 of the first expansion or the second expansion.

[00606] TALEN construction and electroporation may be carried out according to methods described by Menger, et al., *Cancer Res.*, 2016 Apr 15; 76(8):2087-93, Gautron, et al., *et al.*, *Molecular Therapy: Nucleic Acids* 2017, 9:312-321, or U.S. Patent No. 9,458,439, the disclosures of each of which are incorporated by reference herein. TALEN targeting the PD-1 gene will be produced, *e.g.*, using a solid phase assembly method described by Daboussi et al., *Nat Commun* 2014; 5:3831, which is incorporated by reference herein, or may be obtained commercially from Trilink Biotechnologies or other providers as described in Gautron, et al., *et al.*, *Molecular Therapy: Nucleic Acids* 2017, 9:312-321. The procedure

described in Gautron, et al., *et al.*, *Molecular Therapy: Nucleic Acids* 2017, 9:312-321, and described elsewhere herein, for activation of TILs, may be employed, wherein TILs are activated using Dynabeads human T-cell activator CD3/CD28 beads (available commercially from Invitrogen) at ratio of 1:1 $CD3^+$ bead:cell or human T-cell activator CD3/CD28 antibody complexes such as Immunocult CD3/CD28 activator (available commercially from StemCell Technologies). A total of 5×10^6 TILs/180 μL of BTX cytoporation medium T may be mixed with about 10 to 20 μg of the *in vitro* transcribed TALEN mRNA (mMESSAGE mMACHINE T7 kit; Ambion), before electroporation using an Agile Pulse BTX system (Harvard Apparatus). Electroporated cells may be expanded by pre-REP and REP methods described elsewhere herein. Post-REP TILs will be assessed for cell viability and fold expansion (cell counter) and PD-1 knockdown (flow and qPCR). The target results are >80% viability or within 10 % of a "no electroporation" (NE) control; >70% transfection; fold expansion of TILs to within 30% of NE; and >50% PD-1 knockout. Electroporation methods known in the art, such as those described in U.S. Patent Nos. 6,010,613 and 6,078,490, the disclosures of which are incorporated by reference herein, may be used.

[00607] Pulsed electroporation optimization may be performed using methods described herein or as follows. A first set of experiments were performed on TILs in order to determine a voltage range in which cells could be transfected. Five different programs were tested:

	Group 1				Group 2				Group 3			
Program	Pulses	V	Duration (ms)	Interval (ms)	Pulses	V	Duration (ms)	Interval (ms)	Pulses	V	Duration (ms)	Interval (ms)
1	1	600	0.1	0.2	1	600	0.1	100	4	130	0.2	2
2	1	900	0.1	0.2	1	900	0.1	100	4	130	0.2	2
3	1	1200	0.1	0.2	1	1200	0.1	100	4	130	0.2	2
4	1	1200	0.1	10	1	900	0.1	100	4	130	0.2	2
5	1	900	0.1	20	1	600	0,1	100	4	130	0.2	2

[00608] TILs may be electroporated in 0.4 cm gap cuvette (on the order of about 10^6 cells/mL) with about 20 μg of plasmids encoding GFP and control plasmids pUC using the different electroporation programs. About 24 hours post electroporation, GFP expression was analyzed in electroporated cells by flow cytometry to determine the efficiency of transfection. The minimal voltage required for plasmid electroporation in TILs has been previously reported in International Patent Application Publication No. WO 2014/184744 and

U.S Patent Application Publication No. US 2013/0315884 A1, the disclosures of which are incorporated by reference herein, and program 3 and 4 each allow for an efficient TALEN incorporation process for TILs.

[00609] The TALEN construct shown in Figure 23 targets exon 2 of the Pdcd1 gene, as shown, and may be used for the inactivation of PD1.

[00610] 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.

[00611] 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.

[00612] 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.

[00613] 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 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 patient by processing a tumor sample obtained from the patient into multiple tumor fragments;
   (b) adding tumor fragments into a closed system, wherein the tumor fragments are from a tumor resected from a patient;
   (c) 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, 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, optionally 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, 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 the therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system;
   (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; and
   (g) at any time during the method, gene-editing at least a portion of the TILs.
2. The method according to claim 1, further comprising the step of cryopreserving the infusion bag comprising the harvested TIL population in step (f) using a cryopreservation process.

3. The method according to claim 1, wherein the cryopreservation process is performed using a 1:1 ratio of harvested TIL population to cryopreservation media.

4. The method according to claim 1, wherein the antigen-presenting cells are peripheral blood mononuclear cells (PBMCs).

5. The method according to claim 4, wherein the PBMCs are irradiated and allogeneic.

6. The method according to claim 4, wherein the PBMCs are added to the cell culture on any of days 9 through 14 in step (d).

7. The method according to claim 1, wherein the antigen-presenting cells are artificial antigen-presenting cells.

8. The method according to claim 1, wherein the harvesting in step (e) is performed using a membrane-based cell processing system.

9. The method according to claim 1, wherein the harvesting in step (e) is performed using a LOVO cell processing system.

10. The method according to claim 1, wherein the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3.

11. The method according to claim 1, wherein the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 mm^3 to about 1500 mm^3.

12. The method according to claim 9, wherein the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3.

13. The method according to claim 1, wherein the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams.

14. The method according to claim 1, wherein the cell culture medium is provided in a container selected from the group consisting of a G-container and a Xuri cellbag.

15. The method according to claim 1, wherein the cell culture medium in step (d) further comprises IL-15 and/or IL-21.

16. The method according to claim any of the preceding claims, wherein the IL-2 concentration is about 10,000 IU/mL to about 5,000 IU/mL.

17. The method according to claim 15, wherein the IL-15 concentration is about 500 IU/mL to about 100 IU/mL.

18. The method according to claim 1, wherein the IL-21 concentration is about 20 IU/mL to about 0.5 IU/mL.

19. The method according to claim 1, wherein the infusion bag in step (f) is a HypoThermosol-containing infusion bag.

20. The method according to claim 3, wherein the cryopreservation media comprises dimethlysulfoxide (DMSO).

21. The method according to claim 17, wherein the wherein the cryopreservation media comprises 7% to 10% DMSO.

22. The method according to claim 1, wherein the first period in step (c) and the second period in step (e) are each individually performed within a period of 10 days, 11 days, or 12 days.

23. The method according to claim 1, wherein the first period in step (c) and the second period in step (e) are each individually performed within a period of 11 days.

24. The method according to claim 1, wherein steps (a) through (f) are performed within a period of about 10 days to about 22 days.

25. The method according to claim 1, wherein steps (a) through (f) are performed within a period of about 20 days to about 22 days.

26. The method according to claim 1, wherein steps (a) through (f) are performed within a period of about 15 days to about 20 days.

27. The method according to claim 1, wherein steps (a) through (f) are performed within a period of about 10 days to about 20 days.

28. The method according to claim 1, wherein steps (a) through (f) are performed within a period of about 10 days to about 15 days.

29. The method according to claim 1, wherein steps (a) through (f) are performed in 22 days or less.

30. The method according to claim 1, wherein steps (a) through (f) are performed in 20 days or less.

31. The method according to claim 1, wherein steps (a) through (f) are performed in 15 days or less.

32. The method according to claim 1, wherein steps (a) through (f) are performed in 10 days or less.

33. The method according to claim 2, wherein steps (a) through (f) and cryopreservation are performed in 22 days or less.

34. The method according to any one of claims 1 to 33, wherein the therapeutic population of TILs harvested in step (e) comprises sufficient TILs for a therapeutically effective dosage of the TILs.

35. The method according to claim 34, wherein the number of TILs sufficient for a therapeutically effective dosage is from about 2.3×10^{10} to about 13.7×10^{10}.

36. The method according to any one of claims 1 to 35, wherein steps (b) through (e) are performed in a single container, wherein performing steps (b) through (e) in a single container results in an increase in TIL yield per resected tumor as compared to performing steps (b) through (e) in more than one container.

37. The method according to any one of claims 1 to 36, wherein the antigen-presenting cells are added to the TILs during the second period in step (d) without opening the system.

38. The method according to any one of claims 1 to 37, wherein the third population of TILs in step (d) provides for increased efficacy, increased interferon-gamma production, increased polyclonality, increased average IP-10, and/or increased average MCP-1 when administered to a subject.

39. The method according to any one of claims 1 to 38, wherein the third population of TILs in step (d) provides for at least a five-fold or more interferon-gamma production when administered to a subject.

40. The method according to any one of claims 1 to 39, wherein the third population of TILs in step (d) 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 effector T cells and/or central memory T cells in the therapeutic population of TILs exhibit one or more characteristics selected from the group consisting of expressing CD27+, expressing CD28+, longer telomeres, increased CD57 expression, and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

41. The method according to any one of claims 1 to 40, wherein the effector T cells and/or central memory T cells obtained from the third population of TILs exhibit increased CD57 expression and decreased CD56 expression relative to effector T cells and/or central memory T cells obtained from the second population of cells.

42. The method according to any one of claims 1 to 41, wherein the risk of microbial contamination is reduced as compared to an open system.

43. The method according to any one of claims 1 to 42, wherein the TILs from step (g) are infused into a patient.

44. The method according to any one of claims 1 to 43, wherein the multiple fragments comprise about 4 fragments.

45. The method according to any one of claims 1 to 44, wherein the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

46. The method according to claim 45, wherein and the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

47. The method according to claim 45, wherein and the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

48. The method according to any of claims 1-47, wherein the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

49. The method according to any of claims 1-47, wherein the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

50. The method according to any of claims 1-47, wherein the gene-editing is carried out after the first expansion and before the second expansion.

51. The method according to any of claims 1-47, wherein the gene-editing is carried out before step (c), before step (d), or before step (e).

52. The method according to any of claims 1-47, wherein the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

53. The method according to any of claims 1-47, wherein the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

54. The method according to any of claims 1-47, wherein the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

55. The method according to any of claims 1-54, wherein the gene-editing 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.

56. The method according to claim 55, wherein said one or more immune checkpoint genes is/are selected from the group comprising 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.

57. The method according to claim 55, wherein said one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

58. The method according to any of claims 1-54, wherein the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

59. The method according to any of claims 1-58, wherein the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

60. The method according to any of claims 1-58, wherein the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

61. The method according to any of claims 1-58, wherein the gene-editing comprises a CRISPR method.

62. The method according to claim 61, wherein the CRISPR method is a CRISPR/Cas9 method.

63. The method according to any of claims 1-58, wherein the gene-editing comprises a TALE method.

64. The method according to any of claims 1-58, wherein the gene-editing comprises a zinc finger method.

65. A method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:
    (a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the patient 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, and optionally OKT-3, 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, optionally 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, 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 the 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) optionally cryopreserving the infusion bag comprising the harvested TIL population from step (f) using a cryopreservation process;

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

(i) at any time during the method steps (a)-(f), gene-editing at least a portion of the TILs.

66. The method according to claim 65, wherein the therapeutic population of TILs harvested in step (e) comprises sufficient TILs for administering a therapeutically effective dosage of the TILs in step (h).

67. The method according to claim 66, wherein the number of TILs sufficient for administering a therapeutically effective dosage in step (h) is from about 2.3×10^{10} to about 13.7×10^{10}.

68. The method according to claim 67, wherein the antigen presenting cells (APCs) are PBMCs.

69. The method according to claim 68, wherein the PBMCs are added to the cell culture on any of days 9 through 14 in step (d).

70. The method according to any of claims 65 to 69, wherein prior to administering a therapeutically effective dosage of TIL cells in step (h), a non-myeloablative lymphodepletion regimen has been administered to the patient.

71. The method according to claim 70, where 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.

72. The method according to any of claims 65 to 71, further comprising the step of treating the patient with a high-dose IL-2 regimen starting on the day after administration of the TIL cells to the patient in step (h).

73. The method according to claim 72, wherein 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.

74. The method according to any of claims 65-73, wherein the third population of TILs in step (d) 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 effector T cells and/or central memory T cells in the therapeutic population of TILs exhibit one or more characteristics selected from the group consisting of expressing CD27+, expressing CD28+, longer telomeres, increased CD57 expression, and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

75. The method according to any of claims 65-74, wherein the effector T cells and/or central memory T cells in the therapeutic population of TILs exhibit increased CD57 expression and decreased CD56 expression relative to effector T cells and/or central memory T cells obtained from the second population of cells.

76. The method according to any of claims 65-75, wherein 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)), renal cancer, and renal cell carcinoma.

77. The method according to any of claims 65-76, wherein the cancer is selected from the group consisting of melanoma, HNSCC, cervical cancers, and NSCLC.

78. The method according to any of claims 65-77, wherein the cancer is melanoma.

79. The method according to any of claims 65-78, wherein the cancer is HNSCC.

80. The method according to any of claims 65-79, wherein the cancer is a cervical cancer.

81. The method according to any of claims 65-80, wherein the cancer is NSCLC.

82. The method according to any one of claims 65 to 81, wherein the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

83. The method according to claim 82, wherein and the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

84. The method according to claim 82, wherein and the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

85. The method according to any of claims 65-84, wherein the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

86. The method according to any of claims 65-84, wherein the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

87. The method according to any of claims 65-84, wherein the gene-editing is carried out after the first expansion and before the second expansion.

88. The method according to any of claims 65-84, wherein the gene-editing is carried out before step (c), before step (d), or before step (e).

89. The method according to any of claims 65-88, wherein the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

90. The method according to any of claims 65-88, wherein the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

91. The method according to any of claims 65-88, wherein the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

92. The method according to any of claims 65-91, wherein the gene-editing 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.

93. The method according to claim 92, wherein said one or more immune checkpoint genes is/are selected from the group comprising 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.

94. The method according to claim 92, wherein said one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

95. The method according to any of claims 65-91, wherein the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

96. The method according to any of claims 65-95, wherein the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

97. The method according to any of claims 65-95, wherein the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

98. The method according to any of claims 65-95, wherein the gene-editing comprises a CRISPR method.

99. The method according to claim 98, wherein the CRISPR method is a CRISPR/Cas9 method.

100. The method according to any of claims 65-95, wherein the gene-editing comprises a TALE method.

101. The method according to any of claims 65-95, wherein the gene-editing comprises a zinc finger method.

102. A method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

(a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;

(b) 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, 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 (a) to step (b) occurs without opening the system;

(c) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally 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, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;

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

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

(f) at any time during the method, gene-editing at least a portion of the TILs.

103. The method according to claim 102, wherein the therapeutic population of TILs harvested in step (d) comprises sufficient TILs for a therapeutically effective dosage of the TILs.

104. The method according to claim 103, where the number of TILs sufficient for a therapeutically effective dosage is from about 2.3×10^{10} to about 13.7×10^{10}.

105. The method according to claim 104, further comprising the step of cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

106. The method according to claim 105, wherein the cryopreservation process is performed using a 1:1 ratio of harvested TIL population to cryopreservation media.

107. The method according to claim 102, wherein the antigen-presenting cells are peripheral blood mononuclear cells (PBMCs).

108. The method according to claim 107, wherein the PBMCs are irradiated and allogeneic.

109. The method according to claim 108, wherein the PBMCs are added to the cell culture on any of days 9 through 14 in step (c).

110. The method according to claim 102, wherein the antigen-presenting cells are artificial antigen-presenting cells.

111. The method according to claim 102, wherein the harvesting in step (d) is performed using a LOVO cell processing system.

112. The method according to claim 62, wherein the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3.

113. The method according to claim 102, wherein the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 mm^3 to about 1500 mm^3.

114. The method according to claim 103, wherein the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3.

115. The method according to claim 102, wherein the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams.

116. The method according to claim 102, wherein the multiple fragments comprise about 4 fragments.

117. The method according to claim 102, wherein the second cell culture medium is provided in a container selected from the group consisting of a G-container and a Xuri cellbag.

118. The method according to claim 102, wherein the infusion bag in step (e) is a HypoThermosol-containing infusion bag.

119. The method according to claim 102, wherein the first period in step (b) and the second period in step (c) are each individually performed within a period of 10 days, 11 days, or 12 days.

120. The method according to claim 102, wherein the first period in step (b) and the second period in step (c) are each individually performed within a period of 11 days.

121. The method according to claim 102, wherein steps (a) through (e) are performed within a period of about 10 days to about 22 days.

122. The method according to claim 102, wherein steps (a) through (e) are performed within a period of about 10 days to about 20 days.

123. The method according to claim 102, wherein steps (a) through (e) are performed within a period of about 10 days to about 15 days.

124. The method according to claim 102, wherein steps (a) through (e) are performed in 22 days or less.

125. The method according to claim 105, wherein steps (a) through (e) and cryopreservation are performed in 22 days or less.

126. The method according to any one of claims 102 to 125, wherein steps (b) through (e) are performed in a single container, wherein performing steps (b) through (e) in a single container results in an increase in TIL yield per resected tumor as compared to performing steps (b) through (e) in more than one container.

127. The method according to any one of claims 102 to 126, wherein the antigen-presenting cells are added to the TILs during the second period in step (c) without opening the system.

128. The method according to any one of claims 102 to 127, wherein the third population of TILs in step (d) 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 effector T cells and/or central memory T cells obtained in the therapeutic population of TILs exhibit one or more characteristics selected from the group consisting of expressing CD27+, expressing CD28+, longer telomeres, increased CD57 expression, and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

129. The method according to any one of claims 102 to 128, wherein the effector T cells and/or central memory T cells obtained in the therapeutic population of TILs exhibit increased CD57 expression and decreased CD56 expression relative to effector T cells, and/or central memory T cells obtained from the second population of cells.

130. The method according to any one of claims 102 to 129, wherein the risk of microbial contamination is reduced as compared to an open system.

131. The method according to any one of claims 102 to 130, wherein the TILs from step (e) are infused into a patient.

132. The method according to any of claims 102 to 131 wherein the closed container comprises a single bioreactor.

133. The method according to claim 132, wherein the closed container comprises a G-REX-10.

134. The method according to claim 132, wherein the closed container comprises a G-REX -100.

135. The method according to any one of claims 102 to 134, wherein at step (d) the antigen presenting cells (APCs) are added to the cell culture of the second population of TILs at a APC:TIL ratio of 25:1 to 100:1.

136. The method according to claim 135, wherein the cell culture has a ratio of 2.5×10^9 APCs to 100×10^6 TILs.

137. The method according to any one of claims 102 to 134, wherein at step (c) the antigen presenting cells (APCs) are added to the cell culture of the second population of TILs at a APC:TIL ratio of 25:1 to 100:1.

138. The method according to claim 137, wherein the cell culture has ratio of 2.5×10^9 APCs to 100×10^6 TILs.

139. The method according to any one of claims 102-138, wherein the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

140. The method according to claim 139, wherein and the gene-editing is carried out after the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

141. The method according to claim 139, wherein and the gene-editing is carried out before the 4-1BB agonist and/or the OX40 agonist is introduced into the cell culture medium.

142. The method according to any of claims 102-141, wherein the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

143. The method according to any of claims 102-141, wherein the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

144. The method according to any of claims 102-141, wherein the gene-editing is carried out after the first expansion and before the second expansion.

145. The method according to any of claims 102-141, wherein the gene-editing is carried out before step (b), before step (c), or before step (d).

146. The method according to any of claims 102-145, wherein the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out before the OKT-3 is introduced into the cell culture medium.

147. The method according to any of claims 102-145, wherein the cell culture medium comprises OKT-3 during the first expansion and/or during the second expansion, and the gene-editing is carried out after the OKT-3 is introduced into the cell culture medium.

148. The method according to any of claims 102-145, wherein the cell culture medium comprises OKT-3 beginning on the start day of the first expansion, and the gene-editing is carried out after the TILs have been exposed to the OKT-3.

149. The method according to any of claims 102-148, wherein the gene-editing 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.

150. The method according to claim 149, wherein said one or more immune checkpoint genes is/are selected from the group comprising 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.

151. The method according to claim 149, wherein said one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

152. The method according to any of claims 102-148, wherein the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

153. The method according to any of claims 102-152, wherein the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

154. The method according to any of claims 102-152, wherein the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

155. The method according to any of claims 102-152, wherein the gene-editing comprises a CRISPR method.

156. The method according to claim 155, wherein the CRISPR method is a CRISPR/Cas9 method.

157. The method according to any of claims 102-152, wherein the gene-editing comprises a TALE method.

158. The method according to any of claims 102-152, wherein the gene-editing comprises a zinc finger method.

159. A population of expanded TILs for use in the treatment of a subject with cancer, wherein the population of expanded TILs is a third population of TILs obtainable by a method comprising:

(a) obtaining a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the patient 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, and optionally OKT-3, 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, optionally 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, 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 the therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system;

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

(h) at any time during the method, gene-editing at least a portion of the TILs.

160. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the method further comprises one or more of the features recited in any of claims 1 to 158.

161. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist during the first expansion, the second expansion, or both.

162. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing is carried out on TILs from one or more of the first population, the second population, and the third population.

163. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing is carried out on TILs from the first expansion, or TILs from the second expansion, or both.

164. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing is carried out after the first expansion and before the second expansion.

165. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing is carried out before step (c), before step (d), or before step (e)

166. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the population of expanded TILs.

167. The population of TILs for use to treat a subject with cancer according to claim 166, wherein said one or more immune checkpoint genes is/are selected from the group

comprising 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.

168. The population of TILs for use to treat a subject with cancer according to claim 166, wherein said one or more immune checkpoint genes is/are selected from the group comprising PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, and PKA.

169. The population of TILs for use to treat a subject with cancer according to claim 166, wherein the gene-editing causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs, the immune checkpoint gene(s) being selected from the group comprising CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

170. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing comprises the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at said one or more immune checkpoint genes.

171. The population of TILs for use to treat a subject with cancer according to claim 159, wherein the gene-editing comprises one or more methods selected from a CRISPR method, a TALE method, a zinc finger method, and a combination thereof.

172. A cryopreservation composition comprising the population of TILs for use to treat a subject with cancer according to claim 159, a cryoprotectant medium comprising DMSO, and an electrolyte solution.

173. The cryopreservation composition of claim 172 further comprising one or more stabilizers and one or more lymphocyte growth factors.

174. The cryopreservation composition of claim 173, wherein the one or more stabilizers comprise Human Serum Albumin (HSA) and the one or more lymphocyte growth factors comprise IL-2.

175. The cryopreservation composition of claim 174, wherein the cryoprotectant medium comprising DMSO and the electrolyte solution are present in a ratio of about 1.1:1 to about 1:1.1.

176. The cryopreservation composition of claim 174 comprising the population of TILs in an amount of about 1 X 10^6 to about 9 X 10^{14}, the cryoprotectant medium comprising DMSO in an amount of about 30 mL to about 70 mL, the electrolyte solution in an amount of about 30 mL to about 70 mL, HSA in an amount of about 0.1 g to about 1.0 g, and IL-2 in an amount of about 0.001 mg to about 0.005 mg.

177. 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 patient by processing a tumor sample obtained from the patient 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 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 2 to 5 days;

(d) optionally adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

178. The method according to claim 177 comprising performing the first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2, OKT-3 and a 4-1BB agonist antibody, wherein the OKT-3 and the 4-1BB agonist antibody are optionally present in the cell culture medium beginning on Day 0 or Day 1.

179. The method according to claim 177 or claim 178, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CISH, and CBLB, and combinations thereof.

180. The method according to claim 177 or claim 178, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1.

181. The method according to claim 177 or claim 178, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat

(CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of LAG-3.

182. The method according to claim 177 or claim 178, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of TIM-3.

183. The method according to claim 177 or claim 178, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of CISH.

184. The method according to claim 177 or claim 178, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of CBLB.

185. The method according to any of claims 177 to 184, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system.

186. The method according to any of claims 177 to 184, wherein the electroporation step comprises the delivery of a Transcription Activator-Like Effector (TALE) system.

187. The method according to any of claims 177 to 184, wherein the electroporation step comprises the delivery of a zinc finger system.

188. The method according to any of claims 177 to 187, wherein the dimethylsulfoxide-based cryopreservation medium comprises DMSO, an electrolyte solution, optionally HSA, and optionally IL-2.

189. A method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 2 to 5 days;

(d) optionally adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody, optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium; and

(k) administering a therapeutically effective dosage of the harvested TIL population from the infusion bag to the patient;

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3,

TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

190. The method according to claim 189 comprising performing the first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2, OKT-3 and a 4-1BB agonist antibody, wherein the OKT-3 and the 4-1BB agonist antibody are optionally present in the cell culture medium beginning on Day 0 or Day 1.

191. The method according to claim 189 or claim 190, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CISH, and CBLB, and combinations thereof.

192. The method according to claim 189 or claim 190, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1.

193. The method according to claim 189 or claim 190, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of LAG-3.

194. The method according to claim 189 or claim 190, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of TIM-3.

195. The method according to claim 189 or claim 190, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of CISH.

196. The method according to claim 189 or claim 190, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of CBLB.

197. The method according to any of claims 189 to 196, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system.

198. The method according to any of claims 189 to 196, wherein the electroporation step comprises the delivery of a Transcription Activator-Like Effector (TALE) system.

199. The method according to any of claims 189 to 196, wherein the electroporation step comprises the delivery of a zinc finger system.

200. The method according to any of claims 189 to 196, wherein the dimethylsulfoxide-based cryopreservation medium comprises DMSO, an electrolyte solution, optionally HSA, and optionally IL-2.

201. A population of expanded TILs for use in the treatment of a subject with cancer, wherein the population of expanded TILs is a harvested population of TILs obtainable by a method comprising:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient 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 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 2 to 5 days;

(d) optionally adding OKT-3, 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 1 to 3 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 (c) to step (d) occurs without opening the system;

(e) performing a sterile electroporation step on the second population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor;

(f) resting the second population of TILs for about 1 day;

(g) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, optionally OKT-3 antibody,

optionally an OX40 antibody, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7 to 11 days to obtain the third 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 (f) to step (g) occurs without opening the system;

(h) harvesting the therapeutic population of TILs obtained from step (g) to provide a harvested TIL population, wherein the transition from step (g) to step (h) occurs without opening the system, wherein the harvested population of TILs is a therapeutic population of TILs;

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

(j) cryopreserving the harvested TIL population using a dimethylsulfoxide-based cryopreservation medium,

wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, CISH, TGFβR2, PRA, CBLB, BAFF (BR3), and combinations thereof.

202. The population of expanded TILs according to claim 201 comprising performing the first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2, OKT-3 and a 4-1BB agonist antibody, wherein the OKT-3 and the 4-1BB agonist antibody are optionally present in the cell culture medium beginning on Day 0 or Day 1.

203. The population of expanded TILs according to claim 201 or claim 202, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of a molecule selected from the group consisting of PD-1, LAG-3, TIM-3, CISH, and CBLB, and combinations thereof.

204. The population of expanded TILs according to claim 201 or claim 202, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short

Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of PD-1.

205. The population of expanded TILs according to claim 201 or claim 202, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of LAG-3.

206. The population of expanded TILs according to claim 201 or claim 202, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of TIM-3.

207. The population of expanded TILs according to claim 201 or claim 202, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of CISH.

208. The population of expanded TILs according to claim 201 or claim 202, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system, a Transcription Activator-Like Effector (TALE) system, or a zinc finger system for inhibiting the expression of CBLB.

209. The population of expanded TILs according to claim 201 to 208, wherein the electroporation step comprises the delivery of a Clustered Regularly Interspersed Short Palindromic Repeat (CRISPR) system.

210. The population of expanded TILs according to claim 201 to 208, wherein the electroporation step comprises the delivery of a Transcription Activator-Like Effector (TALE) system.

211. The population of expanded TILs according to claim 201 to 208, wherein the electroporation step comprises the delivery of a zinc finger system.

212. The population of expanded TILs according to claim 201 to 211, wherein the dimethylsulfoxide-based cryopreservation medium comprises DMSO, an electrolyte solution, optionally HSA, and optionally IL-2.

213. The population of expanded TILs according to claim 201 to 211, wherein the electroporation step comprises a pulsed electroporation step.

214. The method according to any of claims 1-58, wherein the gene-editing comprises a CRISPR method, and the CRISPR method includes use of a high-fidelity Cas9.