

EXPANSION OF TILS FROM CRYOPRESERVED TUMOR SAMPLES

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This international PCT application claims priority to U.S. Provisional Application No. 62/733,937, filed on September 20, 2018, and U.S. Provisional Application No. 62/879,881, filed on July 29, 2019, each of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[002] The invention described herein relates generally to the expansion of lymphocytes and more particularly, but not exclusively, to expansion of lymphocytes from cryopreserved tumor tissue.

BACKGROUND OF THE INVENTION

[003] Treatment of refractory cancers using adoptive transfer of tumor infiltrating lymphocytes (TILs) represents a potentially powerful approach to treat patients with poor prognoses. Gattinoni, *et al.*, *Nat. Rev. Immunol.* **2006**, *6*, 383-393. Successful immunotherapy requires a large number of TILs; therefore, a robust and reliable process is needed for manufacturing and commercialization. This scaling for commercialization has been a profound challenge because of the legion 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 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. A 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 a REP procedure have produced successful adoptive cell therapy following host immunosuppression in some melanoma patients.

[004] Current TIL manufacturing processes are limited by duration, cost, sterility concerns, and other factors described herein such that the potential to commercialize such processes is severely limited. Among the many limitations of the current processes is the dependence on

fresh tumor tissue to initiate manufacturing, which often necessitates expensive and costly local manufacturing facilities because of the difficulty in shipping fresh tumor longer distances. There is an urgent need to provide TIL manufacturing processes and therapies that are not dependent on fresh tumor tissue and that are appropriate for commercial scale manufacturing and regulatory approval for use in geographically widely dispersed human patients.

SUMMARY OF THE INVENTION

[005] In an embodiment, the invention provides methods of TIL expansion that utilize frozen tumor tissue as the starting TIL source material to produce therapeutic populations of TILs.

[006] In an embodiment, the invention provides a method for cryopreserving tumor tissue for the manufacture of tumor infiltrating lymphocytes (TILs) comprising:

- (i) fragmenting the tumor tissue;
- (ii) incubating the fragments in a cryopreservation medium; and,
- (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

[007] In an embodiment, the invention provides a cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by a process comprising the steps of:

- (i) fragmenting a tumor sample;
- (ii) incubating the fragments in a cryopreservation medium; and,
- (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

[008] In an embodiment, the invention provides a cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by a process comprising the steps of:

- (i) fragmenting a tumor sample;

- (ii) incubating the fragments for about 20 minutes to about 70 minutes at a temperature in the range of about 2°C to about 8°C in a cryopreservation medium comprising about 10% v/v dimethylsulfoxide; and,
- (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

[009] In an embodiment, the invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising incubating the tumor tissue for at least 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing;
- (b) thawing the tumor tissue;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (d) removing at least a plurality of the TILs; and
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0010] In an embodiment, the invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) obtaining tumor tissue from a patient;
- (b) incubating the tumor tissue for at least 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C;
- (c) freezing the tumor tissue;
- (d) storing the tumor tissue in a frozen state;
- (b) thawing the tumor tissue;

- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (d) removing at least a plurality of the TILs; and
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0011] In some embodiments, the storage medium comprises 2 to 12 % v/v (volume:volume) dimethylsulfoxide (DMSO). In some embodiments the storage medium comprises 5% v/v DMSO. In some embodiments the storage medium comprises 10% v/v DMSO. In some embodiments, the storage medium comprises between 5 and 10 % v/v DMSO. In some embodiments the storage medium comprises a percentage of DMSO selected from the group consisting of 1% v/v, 2% v/v, 3% v/v, 4% v/v, 5% v/v, 6% v/v, 7% v/v, 8% v/v, 9% v/v, 10% v/v, 11% v/v, 12% v/v, 13% v/v, 14% v/v, and 15% v/v. In some of the foregoing embodiments, the remainder of the storage medium is an aqueous medium.

[0012] In some embodiments, the storage medium comprises 2 to 12 % w/w (weight:weight) dimethylsulfoxide (DMSO). In some embodiments the storage medium comprises 5% w/w DMSO. In some embodiments the storage medium comprises 10% w/w DMSO. In some embodiments, the storage medium comprises between 5 and 10 % w/w DMSO. In some embodiments the storage medium comprises a percentage of DMSO selected from the group consisting of 1% w/w, 2% w/w, 3% w/w, 4% w/w, 5% w/w, 6% w/w, 7% w/w, 8% w/w, 9% w/w, 10% w/w, 11% w/w, 12% w/w, 13% w/w, 14% w/w, and 15% w/w. In some of the foregoing embodiments, the remainder of the storage medium is an aqueous medium.

[0013] In some embodiments, the tumor tissue is incubated for at least 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for between 30 minutes and about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 45 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range

of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing.

[0014] In some embodiments, the storage medium comprises about 5% v/v DMSO and the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the storage medium comprises about 10% v/v DMSO and the tumor tissue is incubated for at least 30 minutes to about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing.

[0015] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
 - (iv) freezing the vessel; and
 - (v) storing the vessel such that the vessel remains frozen;
- (b) thawing the vessel;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (d) removing at least a plurality of the TILs;
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0016] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (b) placing the tumor tissue in a closable vessel containing a storage medium;
- (c) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
- (d) freezing the vessel;
- (e) storing the vessel such that the vessel remains frozen;
- (d) thawing the vessel;
- (f) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (g) removing at least a plurality of the TILs; and
- (h) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0017] In some embodiments, the fresh tumor tissue is trimmed to between 1.5 mm and 6 mm in diameter and between 1.5 mm and 6 mm in thickness. In yet other embodiments, the fresh tumor tissue is trimmed to about 6 mm × 6 mm × 6 mm.

[0018] In some embodiments, the storage medium further comprises an antibacterial agent. In some embodiments the antibacterial agent is gentamicin. In some embodiments the storage medium comprises gentamicin at a concentration of at least 50 µg/mL. In some embodiments the storage medium comprises an antibacterial agent, an antifungal agent, and combinations thereof. In some embodiments, the antifungal agent is amphotericin B and is present from about 0.25 µg/mL to about 2.5 µg/mL. In some embodiments, the antifungal agent is fungin and is present from about 5 µg/mL to about 50 µg/mL.

[0019] In some embodiments, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) or another suitable medium or solution before performing the freezing step of the method. In some embodiments, the washing comprises at least three serial washes of at least three minutes each, wherein the HBSS is replaced after each wash.

[0020] In some embodiments the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[0021] In some embodiments, the freezing step comprises freezing the vessel at a temperature of about -125 °C to about -195 °C. In some embodiments, the vessel is frozen at a temperature of about -125 °C to about -150 °C; in some embodiments the vessel is frozen at a temperature of about -125 °C to about -145 °C; in yet further embodiments, the vessel is frozen at a temperature of about -135 °C.

[0022] In some embodiments, the method is performed with a fresh tumor sample from a human subject.

[0023] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC), and HPV-positive tumor tissue.

[0024] In some embodiments, the method further comprises a split between the first expansion step (c) and the second expansion step (e). In an embodiment, the split occurs on day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some embodiments, steps (c) through (e) are complete in about 22 days. In yet other embodiments, steps (c) through (e) are complete in 21 days; in some embodiments steps (c) through (e) are complete in about 20 days; and in some embodiments steps (c) through (e) are complete in about 16 days.

[0025] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof are provided, such methods comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (b) thawing the tumor tissue;
- (c) adding the tumor fragments into a closed system;
- (d) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (c) to step (d) occurs without opening the system;
- (e) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to

the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (d) to step (e) occurs without opening the system;

- (f) harvesting the therapeutic population of TILs obtained from step (f), wherein the transition from step (e) to step (f) occurs without opening the system;
- (g) transferring the harvested TIL population from step (f) to an infusion bag, wherein the transfer from step (f) to (g) occurs without opening the system;
- (h) cryopreserving the infusion bag comprising the harvested TIL population from step (g) using a cryopreservation process; and
- (i) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

[0026] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof are provided, such methods comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

- (a) obtaining tumor tissue from the subject;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
- (e) freezing the vessel using the vapor phase of liquid nitrogen;
- (f) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (g) thawing the tumor tissue;
- (h) adding the tumor tissue into a closed system;

- (i) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (h) to step (i) occurs without opening the system;
- (j) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (i) to step (j) occurs without opening the system;
- (k) harvesting the therapeutic population of TILs obtained from step (j), wherein the transition from step (j) to step (k) occurs without opening the system;
- (l) transferring the therapeutic population of TILs harvested in step (k) to an infusion bag, wherein the transfer from step (k) to (l) occurs without opening the system;
- (m) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (l) using a cryopreservation process; and
- (n) administering a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (m) to the subject.

[0027] 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, endometrial cancer, thyroid cancer, colorectal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, melanoma, refractory

melanoma, metastatic melanoma, 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.

[0028] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising incubating the tumor tissue for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing;
- (c) thawing the tumor tissue;
- (d) treating the tumor tissue in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;
- (e) removing at least a plurality of the TILs; and
- (f) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0029] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) incubating the tumor tissue for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C;
- (c) freezing the tumor tissue;
- (d) storing the tumor tissue in a frozen state;
- (e) thawing the tumor tissue;
- (f) treating the tumor tissue in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;

- (g) removing at least a plurality of the TILs; and
- (h) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0030] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 30 minutes;
 - (iv) freezing the vessel; and
 - (v) storing the vessel such that the vessel remains frozen;
- (c) thawing the vessel;
- (d) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (e) removing at least a plurality of the TILs; and
- (f) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[0031] In some embodiments, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing the freezing step of the method. In some embodiments, the washing comprises at least three serial washes of at least three minutes each, wherein the HBSS is replaced after each wash.

[0032] In some embodiments the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[0033] In some embodiments, the freezing step comprises freezing the vessel at a temperature of about -125°C to about -195°C. In some embodiments, the vessel is frozen at a temperature of about -125°C to about -150°C; in some embodiments the vessel is frozen at a temperature of about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at a temperature of about -135°C.

[0034] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC), and HPV-positive tumor tissue.

[0035] In some embodiments, the method further comprises a split between the first expansion (or first culture) step and the second expansion (or second culture) step. In an embodiment, the split occurs on day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some embodiments, steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 22 days. In yet other embodiments, steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 21 days; in some embodiments steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 20 days; and in some embodiments steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 16 days.

[0036] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof are provided, such methods comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

- (a) obtaining tumor tissue from the subject, wherein the tumor tissue comprises TILs;

- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 30 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (c) thawing the tumor tissue;
- (d) adding the tumor tissue into a closed system;
- (e) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (d) to step (e) occurs without opening the system;
- (f) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (e) to step (f) occurs without opening the system;

- (g) harvesting the therapeutic population of TILs obtained from step (f), wherein the transition from step (f) to step (g) occurs without opening the system;
- (h) transferring the therapeutic population of TILs harvested from step (g) to an infusion bag, wherein the transfer from step (g) to (h) occurs without opening the system;
- (i) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (h) using a cryopreservation process; and
- (j) administering a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (i) to the subject.

[0037] In some embodiments, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing the freezing step of the method. In some embodiments, the washing comprises at least three serial washes of at least three minutes each, wherein the HBSS is replaced after each wash.

[0038] In some embodiments the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[0039] In some embodiments, the freezing step comprises freezing the vessel at a temperature of about -125°C to about -195°C. In some embodiments, the vessel is frozen at a temperature of about -125°C to about -150°C; in some embodiments the vessel is frozen at a temperature of about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at a temperature of about -135°C.

[0040] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC), and HPV-positive tumor tissue.

[0041] In some embodiments, the method further comprises a split between the first expansion step and the second expansion step. In an embodiment, the split occurs on day 16. In some embodiments, the first expansion step is complete in about 11 days. In other embodiments, the first expansion step is complete in about 7 days. In yet other embodiments, the first expansion

step is complete in about 7 days and the second expansion step is complete in about 14 days. In some embodiments, steps (e) through (g) are complete in about 22 days. In yet other embodiments, steps (e) through (g) are complete in about 21 days; in some embodiments steps (e) through (g) are complete in about 20 days; and in some embodiments steps (e) through (g) are complete in about 16 days.

[0042] In some embodiments, an expansion method according to the present invention is completed in about 16 days. In some embodiments, an expansion method comprises a first expansion step (a preREP step) and a second expansion step (a REP step). In an embodiment, the first expansion step is from about 3 to about 5 days. In another embodiment, the first expansion step is about 3 days. In another embodiment, the first expansion step is about 5 days. In some embodiments, the second expansion step is from about 11 to about 13 days. In another embodiment, the second expansion step is about 11 days. In another embodiment, the second expansion step is about 13 days. In some embodiments, the first expansion step is about 5 days and the second expansion step is about 11 days. In some embodiments, the first expansion step is about 3 days and the second expansion step is about 13 days.

[0043] In some embodiments, the TILs are split into additional containers during the second expansion step. In some embodiments, the TILs are split on day 5 of the second expansion step. In some embodiments, the TILs are split on day 6 of the second expansion step. In some embodiments, the second expansion step is about 11 days and the TILs are split on day 5 of the second expansion step. In some embodiments, the second expansion step is about 13 days and the TILs are split on day 6 of the second expansion step.

[0044] In some embodiments, the first expansion step is 3 days, the second expansion step is 13 days, and the TILs are split on day 6 of the second expansion step (day 9 of the total process). In some embodiments, the first expansion step is 5 days, the second expansion step is 11 days, and the TILs are split on day 5 of the second expansion step (day 10 of the total process).

[0045] In some embodiments, feeder cells are used during the second expansion step. In an embodiment, in a 1/100th scale process, about $2\text{--}50 \times 10^6$ feeder cells are used during the second expansion step. In another embodiment, about 25×10^6 feeder cells are used. In another embodiment, about 50×10^6 feeder cells are used. In some embodiments, in a full scale process,

about $2\text{--}50 \times 10^9$ feeder cells are used. In some embodiments, in a full scale process, about 2.5×10^9 or about 5×10^9 feeder cells are used. In some embodiments, the feeder cells are PBMCs.

[0046] In some embodiments, the second expansion step is seeded with from about 5×10^6 to about 200×10^6 TIL. In some embodiments, the second expansion step is seeded with about 5% to about 25% of the TILs from the first expansion step. In some embodiments, the second expansion step is seeded with about 10% to about 20% of the TILs from the first expansion step. In some embodiments, the second expansion step is seeded with about 10% of the TILs from the first expansion step.

[0047] Other embodiments include combinations and variations of these compositions and methods.

BRIEF DESCRIPTION OF THE DRAWINGS

[0048] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings.

[0049] **Figure 1:** Depicts the major steps of an embodiment of the 2A process, including the optional cryopreservation of fresh tumor tissue after fragmentation in Step B. The total time from the initiation of the first expansion culture to the end of Step E is about 22 days.

[0050] **Figure 2:** Panels A through C depict the various steps of an embodiment of the TIL manufacturing process, including cryopreservation of fresh tumor fragments allowing later initiation of preREP culture.

[0051] **Figure 3:** Depicts an embodiment of TIL manufacturing, including the cryopreservation of fresh tumor fragments that are then later used to initiate a preREP culture.

[0052] **Figure 4:** Depicts an embodiment of TIL manufacturing, including the cryopreservation of fresh tumor fragments that are then later used to initiate a pre-REP culture.

[0053] **Figure 5:** Generally compares an embodiment of Process 1C to an exemplary embodiment of Process 2A. Process 2A contemplates initiation of pre-REP cultures from either fresh tumor tissue fragments or thawed cryopreserved tumor tissue fragments.

[0054] **Figure 6:** Further compares embodiments of Process 1C to embodiments of Process

2A.

[0055] Figure 7: Depicts an embodiment of the 2A process wherein the REP is initiated “early.”

[0056] Figure 8: Compares the total viable cells derived from a Gen 2A process and a modified Gen 2A process, noted as “Gen 2.2” wherein the pre-REP cultures are initiated with fresh, flash frozen, or frozen following 30 minute or 60 minute incubations in storage medium at 2°C to 8°C. Cell counts are at the end of the pre-REP culture.

[0057] Figure 9: Compares the total viable cells derived from a Gen 2A process and a modified Gen 2A process, noted as “Gen 2.2” wherein the pre-REP cultures are initiated with fresh, flash frozen, or frozen following 30 minute or 60 minute incubations in storage medium at 2°C to 8°C. Cell counts at the end of the REP culture.

[0058] Figure 10: Compares IFN- γ produced by cells derived from a Gen 2A process and a modified Gen 2A process, noted as “Gen 2.2” wherein the pre-REP cultures are initiated with fresh, flash frozen, or frozen following 30 minute or 60 minute incubations in storage medium at 2°C to 8°C.

[0059] Figure 11: Depicts the major steps of a Gen 2 embodiment compared to the major steps of a Gen 2.2 embodiment.

[0060] Figure 12: Depicts the major steps of the 1/100th scale TIL production process.

[0061] Figure 13: Figures 13A-13E depict total viable cells (TVC) for tumor samples processed from 5 different patients and collected during pre-REP. TVC was measured using fresh (Gen2 control) and frozen tumor samples. TVC was recorded on day 11 for fresh tumor samples or day 7 for frozen tumor samples. Figure 13A (patient M1125) shows the only patient whose tumor samples were subjected to different freezing methods: flash freezing in the vapor phase of liquid nitrogen, and incubation at 2-8° Celsius for either 30 or 60 minutes prior to flash freezing in the vapor phase of liquid nitrogen. For the remaining patients S13018, OV8022, T6042, and O8026 (FIGS. 13B-13E, respectively), the tumor samples were incubated at 2-8° for 60 minutes prior to flash freezing. For each patient, tumor samples were sectioned in 3mm diameter fragments and 4-6 fragments were used for culture. Each bar on the graph represents TVC extrapolated to full scale (multiplied by 10). In FIG 13A, frozen, early pre-REP culture yielded about 1% TVC as

compared to fresh, FIG. 13B, about 25% TVC as compared to fresh, FIG. 13C, about 200% as compared to fresh, FIG. 13D, about 96% as compared to fresh, and FIG. 13E, about 3% as compared with fresh. The data indicate that TVC from frozen tumor samples on Day 7 is comparable to TVC from fresh tumor samples on Day 11.

[0062] Figure 14: Figures 14A-14E depict TVC for tumor samples from the same 5 patients as Figure 13, collected during early REP. Cells were harvested on Day 11 (fresh) and Day 7 (frozen) as described in Figure 13. In FIG. 14A, frozen, early REP culture yielded about 86% TVC as compared to fresh, FIG. 14B, about 98% TVC as compared to fresh, FIG. 14C, about 124% as compared to fresh, FIG. 14D, about 286% as compared to fresh, and FIG. 14E, about 167% as compared with fresh. The data indicate that overall yield (measured as TVC) from frozen tumor samples on Day 7 is comparable to TVC from fresh tumor samples on Day 11 during early REP.

[0063] Figure 15: Figure 15 depicts the CD8/CD4 ratio in fresh and frozen tumor samples from 4 patients. Fresh or frozen REP TIL samples were stained with flow cytometry antibodies (CD3-BUV395, CD62LBV421, CD57-PB, CD11c-BV711, CD28-BB515, CD19-BUV563, CCR7-PE, CD123-BV605, CD27PE-CF594, CD14-BV-650, TCRg/d-APC, CD45-PerCP-Cy5.5, CD45RA-A700, CD56-BUV737, CD8-BV786, CD4-PE-Cy7 AND CD16-APC-Vio770). The identity of TIL were determined based on TCR a/b+ or g/d+, monocytes (CD14+), NK (CD56+, CD16+, CD56+/CD16+ and B cells (CD19+) and purity was determined based on CD3+CD45+ cells. Patients M1125 and T6042 demonstrated high CD8 expression, but overall the CD8/CD4 ratio is comparable between fresh and frozen tumor samples.

[0064] Figure 16: Figures 16A-16H depict CD3+CD45+ expression and other marker expression in the same 4 patients as Figure 15. Figures 16A and 16B depict data for patient M1125; Figures 16C and 16D for patient S13018; Figures 16E and 16F for patient OV8022; and Figures 16G and 16H for patient T6042. The data show that TIL purity is comparable between fresh and frozen tumor samples.

[0065] Figure 17: Figures 17A-17C illustrate expression of CD28, CD27, and CD57 in cells expressing CD4 or CD8 from fresh and frozen tumor samples. Figure 17A is for patient M1125; Figure 17B for patient S13018, and Figure 17C for patient T6042. Data show that the differentiation status between fresh and frozen tumor samples is comparable.

[0066] Figure 18: Figures 18A-18C illustrate memory status in fresh and frozen tumor samples in cells expressing CD4 or CD8. Figure 18A is for patient M1125; Figure 18B for patient S13018, and Figure 18C for patient T6042. Data show that the memory status between fresh and frozen tumor samples is comparable. TN: Naïve (CD45RA+CCR7+), TCM: Central memory (CD45RA-CCR7+), TEM: Effector memory (CD45RA-CCR7-), TEMRA/TEFF: RA+ Effector memory/Effectors (CD45RA+CCR7), Central Memory/Activated cells (CD45RA+CD62L+). Bar graph presented are percentage positive CD45+/- or CCR7 +/- or CD62L +/- when gated on CD4+ or CD8+.

[0067] Figure 19: Figures 19A-19C illustrate activation/exhaustion markers in fresh and frozen tumor samples in cells expressing CD4 (top panel) or CD8 (bottom panel) in 3 patients: M1125 (FIG. 19A); S13018 (FIG. 19B); and T6042 (FIG. 19C). Activation and exhaustion of REP TIL was determined by multicolor flow cytometry. Tumor tissue was stained with antibodies (CD3-BUV395, PD-1-BV421, 2B4/CD244-PB, CD8-BB515, CD25-BUV563, BTLA-PE, KLRG1-PE-Dazzle 594, TIM-3-BV650, CD194/CCR4-APC, CD4-VioGreen, TIGIT-PerCP-eFluor 710, CD183-BV711, CD69-APC-R700, CD95-BUV737, CD127-PE-Cy7, CD103-BV786, LAG-3-APC-eFluor 780) and expression was measured using flow cytometry. Overall, TIL activation/exhaustion status is comparable between fresh and frozen tumor samples.

[0068] Figure 20: Figures 20A-20C illustrate IFN γ function in fresh and frozen tumor samples for 3 patients: M1125 (FIG. 20A); S13018 (FIG. 20B); and T6042 (FIG. 20C). 1×10^5 REP TIL were plated in anti-CD3 plates for 24 hours. Culture supernatants were collected and measured for IFN γ cytokine using an IFN γ Qantikine ELISA kit. Bar graph represent here are Mean + SD of triplicate measurement. Student 't' test was used to calculate statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Overall, the data demonstrate that IFN γ function is higher in frozen tumor samples.

[0069] Figure 21: Figures 21A-21C illustrate Granzyme B (GzmB) function in fresh and frozen tumor samples for 3 patients: M1125 (FIG. 21A); S13018 (FIG. 21B); and T6042 (FIG. 21C). 1×10^5 REP TIL were plated in anti-CD3 plates for 24 hours. Culture supernatants were collected and measured for Granzyme B using a Duo set ELISA kit. Bar graph represent here are Mean + SD of triplicate measurement. Student 't' test was used to calculate statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Overall, the data demonstrate that Granzyme B (GzmB) function is comparable/higher in frozen tumor samples.

[0070] Figure 22: Figures 22A-22C illustrate CD107A expression in fresh and frozen tumor samples for 3 patients: M1125 (FIG 22A); S13018 (FIG. 22B); and T6042 (FIG. 22C). 2×10^5 REP TIL were stimulated with or without stimulation of PMA/IO for 2 hours, then stained for CD107A-APC700 and analyzed by flow cytometry. Bar graph represents CD107A positive cells gated on CD8 or CD4. Overall, the data demonstrate that CD107A expression is comparable between fresh and frozen tumor samples.

[0071] Figure 23: Figures 23A-23C illustrate telomere length relative to 1301 cells in fresh and frozen tumor samples for 3 patients: M1125 (FIG 23A); S13018 (FIG. 23B); and T6042 (FIG. 23C). Flow-FISH was performed using Dako/Agilent Pathology Solutions (Telomere PNA Kit/FITC for Flow Cytometry) kit and the manufacturer's instructions were followed to measure the average length of the Telomere repeat. 1301 T-cell leukemia cell line (SigmaAldrich, St. Louis, MO)) was used as an internal reference standard in each assay. Individual TIL were counted and mixed with 1301 cells at a 1:1 cell ratio. 2×10^6 TIL were mixed with 2×10^6 1301 cells, in situ hybridization was performed using FITC-conjugated Telomere PNA probe (FITC-00-CCCTAA-CCC-TAA-CCC-TAA) and analyzed using Flow cytometry (BD canto II). Telomere fluorescence of the test sample was expressed as a percentage of the geometric mean fluorescence (fl) of the 1301 cells per the following formula: Relative telomere length = $[(\text{mean FITC fl test cells w/ probe} - \text{mean FITC fl test cells w/o probe}) \times \text{DNA index of 1301 cells} \times 100] / [(\text{mean FITC fl 1301 cells w/probe} - \text{mean FITC fl 1301 cells w/o probe}) \times \text{DNA index of test cells}]$. The data indicate that telomere length is comparable or better in frozen tumor samples than fresh tumor samples.

[0072] Figure 24: Figure 24 is a flow chart illustrating certain embodiments of the expansion process for TILs from fresh and frozen tumor fragments: fresh method ("GEN2"), early-REP method 1 ("ER-1"), and early-REP method 2 ("ER-2").

[0073] Figure 25: Figure 25 illustrates structures I-A and I-B as comprising 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), and is then used to link two of the trivalent proteins together through disulfide bonds (small elongated ovals), stabilizing the structure and providing an agonist capable of bringing together the intracellular signaling domains of the six receptors and signaling proteins to form a signaling complex.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

- [0074] SEQ ID NO:1 is the amino acid sequence of the heavy chain of muromonab.
- [0075] SEQ ID NO:2 is the amino acid sequence of the light chain of muromonab.
- [0076] SEQ ID NO:3 is the amino acid sequence of a recombinant human IL-2 protein.
- [0077] SEQ ID NO:4 is the amino acid sequence of aldesleukin.
- [0078] SEQ ID NO:5 is the amino acid sequence of a recombinant human IL-4 protein.
- [0079] SEQ ID NO:6 is the amino acid sequence of a recombinant human IL-7 protein.
- [0080] SEQ ID NO:7 is the amino acid sequence of a recombinant human IL-15 protein.
- [0081] SEQ ID NO:8 is the amino acid sequence of a recombinant human IL-21 protein.
- [0082] SEQ ID NO:9 is the amino acid sequence of human 4-1BB.
- [0083] SEQ ID NO:10 is the amino acid sequence of murine 4-1BB.
- [0084] SEQ ID NO:11 is the heavy chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0085] SEQ ID NO:12 is the light chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0086] SEQ ID NO:13 is the heavy chain variable region (V_H) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0087] SEQ ID NO:14 is the light chain variable region (V_L) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0088] SEQ ID NO:15 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0089] SEQ ID NO:16 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0090] SEQ ID NO:17 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).
- [0091] SEQ ID NO:18 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody

utomilumab (PF-05082566).

[0092] SEQ ID NO:19 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0093] SEQ ID NO:20 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0094] SEQ ID NO:21 is the heavy chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0095] SEQ ID NO:22 is the light chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0096] SEQ ID NO:23 is the heavy chain variable region (V_H) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0097] SEQ ID NO:24 is the light chain variable region (V_L) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0098] SEQ ID NO:25 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0099] SEQ ID NO:26 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00100] SEQ ID NO:27 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00101] SEQ ID NO:28 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00102] SEQ ID NO:29 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00103] SEQ ID NO:30 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00104] SEQ ID NO:31 is an Fc domain for a TNFRSF agonist fusion protein.

[00105] SEQ ID NO:32 is a linker for a TNFRSF agonist fusion protein.

- [00106] SEQ ID NO:33 is a linker for a TNFRSF agonist fusion protein.
- [00107] SEQ ID NO:34 is a linker for a TNFRSF agonist fusion protein.
- [00108] SEQ ID NO:35 is a linker for a TNFRSF agonist fusion protein.
- [00109] SEQ ID NO:36 is a linker for a TNFRSF agonist fusion protein.
- [00110] SEQ ID NO:37 is a linker for a TNFRSF agonist fusion protein.
- [00111] SEQ ID NO:38 is a linker for a TNFRSF agonist fusion protein.
- [00112] SEQ ID NO:39 is a linker for a TNFRSF agonist fusion protein.
- [00113] SEQ ID NO:40 is a linker for a TNFRSF agonist fusion protein.
- [00114] SEQ ID NO:41 is a linker for a TNFRSF agonist fusion protein.
- [00115] SEQ ID NO:42 is an Fc domain for a TNFRSF agonist fusion protein.
- [00116] SEQ ID NO:43 is a linker for a TNFRSF agonist fusion protein.
- [00117] SEQ ID NO:44 is a linker for a TNFRSF agonist fusion protein.
- [00118] SEQ ID NO:45 is a linker for a TNFRSF agonist fusion protein.
- [00119] SEQ ID NO:46 is a 4-1BB ligand (4-1BBL) amino acid sequence.
- [00120] SEQ ID NO:47 is a soluble portion of 4-1BBL polypeptide.
- [00121] SEQ ID NO:48 is a heavy chain variable region (V_H) for the 4-1BB agonist antibody 4B4-1-1 version 1.
- [00122] SEQ ID NO:49 is a light chain variable region (V_L) for the 4-1BB agonist antibody 4B4-1-1 version 1.
- [00123] SEQ ID NO:50 is a heavy chain variable region (V_H) for the 4-1BB agonist antibody 4B4-1-1 version 2.
- [00124] SEQ ID NO:51 is a light chain variable region (V_L) for the 4-1BB agonist antibody 4B4-1-1 version 2.
- [00125] SEQ ID NO:52 is a heavy chain variable region (V_H) for the 4-1BB agonist antibody H39E3-2.

[00126] SEQ ID NO:53 is a light chain variable region (V_L) for the 4-1BB agonist antibody H39E3-2.

[00127] SEQ ID NO:54 is the amino acid sequence of human OX40.

[00128] SEQ ID NO:55 is the amino acid sequence of murine OX40.

[00129] SEQ ID NO:56 is the heavy chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00130] SEQ ID NO:57 is the light chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00131] SEQ ID NO:58 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00132] SEQ ID NO:59 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00133] SEQ ID NO:60 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00134] SEQ ID NO:61 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00135] SEQ ID NO:62 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00136] SEQ ID NO:63 is the light chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00137] SEQ ID NO:64 is the light chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00138] SEQ ID NO:65 is the light chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00139] SEQ ID NO:66 is the heavy chain for the OX40 agonist monoclonal antibody 11D4.

[00140] SEQ ID NO:67 is the light chain for the OX40 agonist monoclonal antibody 11D4.

[00141] SEQ ID NO:68 is the heavy chain variable region (V_H) for the OX40 agonist

monoclonal antibody 11D4.

[00142] SEQ ID NO:69 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 11D4.

[00143] SEQ ID NO:70 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

[00144] SEQ ID NO:71 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

[00145] SEQ ID NO:72 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

[00146] SEQ ID NO:73 is the light chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

[00147] SEQ ID NO:74 is the light chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

[00148] SEQ ID NO:75 is the light chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

[00149] SEQ ID NO:76 is the heavy chain for the OX40 agonist monoclonal antibody 18D8.

[00150] SEQ ID NO:77 is the light chain for the OX40 agonist monoclonal antibody 18D8.

[00151] SEQ ID NO:78 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 18D8.

[00152] SEQ ID NO:79 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 18D8.

[00153] SEQ ID NO:80 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody 18D8.

[00154] SEQ ID NO:81 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

[00155] SEQ ID NO:82 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

[00156] SEQ ID NO:83 is the light chain CDR1 for the OX40 agonist monoclonal antibody 18D8.

[00157] SEQ ID NO:84 is the light chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

[00158] SEQ ID NO:85 is the light chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

[00159] SEQ ID NO:86 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody Hu119-122.

[00160] SEQ ID NO:87 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody Hu119-122.

[00161] SEQ ID NO:88 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.

[00162] SEQ ID NO:89 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

[00163] SEQ ID NO:90 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

[00164] SEQ ID NO:91 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.

[00165] SEQ ID NO:92 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

[00166] SEQ ID NO:93 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

[00167] SEQ ID NO:94 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody Hu106-222.

[00168] SEQ ID NO:95 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody Hu106-222.

[00169] SEQ ID NO:96 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody

Hu106-222.

[00170] SEQ ID NO:97 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

[00171] SEQ ID NO:98 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

[00172] SEQ ID NO:99 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.

[00173] SEQ ID NO:100 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

[00174] SEQ ID NO:101 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

[00175] SEQ ID NO:102 is an OX40 ligand (OX40L) amino acid sequence.

[00176] SEQ ID NO:103 is a soluble portion of OX40L polypeptide.

[00177] SEQ ID NO:104 is an alternative soluble portion of OX40L polypeptide.

[00178] SEQ ID NO:105 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 008.

[00179] SEQ ID NO:106 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 008.

[00180] SEQ ID NO:107 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 011.

[00181] SEQ ID NO:108 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 011.

[00182] SEQ ID NO:109 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 021.

[00183] SEQ ID NO:110 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 021.

[00184] SEQ ID NO:111 is the heavy chain variable region (V_H) for the OX40 agonist

monoclonal antibody 023.

[00185] SEQ ID NO:112 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 023.

[00186] SEQ ID NO:113 is the heavy chain variable region (V_H) for an OX40 agonist monoclonal antibody.

[00187] SEQ ID NO:114 is the light chain variable region (V_L) for an OX40 agonist monoclonal antibody.

[00188] SEQ ID NO:115 is the heavy chain variable region (V_H) for an OX40 agonist monoclonal antibody.

[00189] SEQ ID NO:116 is the light chain variable region (V_L) for an OX40 agonist monoclonal antibody.

[00190] SEQ ID NO:117 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00191] SEQ ID NO:118 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00192] SEQ ID NO:119 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00193] SEQ ID NO:120 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00194] SEQ ID NO:121 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00195] SEQ ID NO:122 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00196] SEQ ID NO:123 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00197] SEQ ID NO:124 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00198] SEQ ID NO:125 is the heavy chain variable region (V_H) for an OX40 agonist monoclonal antibody.

[00199] SEQ ID NO:126 is the light chain variable region (V_L) for an OX40 agonist monoclonal antibody.

[00200] SEQ ID NO:127 is the heavy chain amino acid sequence of the PD-1 inhibitor nivolumab.

[00201] SEQ ID NO:128 is the light chain amino acid sequence of the PD-1 inhibitor nivolumab.

[00202] SEQ ID NO:129 is the heavy chain variable region (V_H) amino acid sequence of the PD-1 inhibitor nivolumab.

[00203] SEQ ID NO:130 is the light chain variable region (V_L) amino acid sequence of the PD-1 inhibitor nivolumab.

[00204] SEQ ID NO:131 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

[00205] SEQ ID NO:132 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

[00206] SEQ ID NO:133 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

[00207] SEQ ID NO:134 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

[00208] SEQ ID NO:135 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

[00209] SEQ ID NO:136 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

[00210] SEQ ID NO:137 is the heavy chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00211] SEQ ID NO:138 is the light chain amino acid sequence of the PD-1 inhibitor

pembrolizumab.

[00212] SEQ ID NO:139 is the heavy chain variable region (V_H) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00213] SEQ ID NO:140 is the light chain variable region (V_L) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00214] SEQ ID NO:141 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00215] SEQ ID NO:142 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00216] SEQ ID NO:143 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00217] SEQ ID NO:144 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00218] SEQ ID NO:145 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00219] SEQ ID NO:146 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00220] SEQ ID NO:147 is the heavy chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00221] SEQ ID NO:148 is the light chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00222] SEQ ID NO:149 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00223] SEQ ID NO:150 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00224] SEQ ID NO:151 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00225] SEQ ID NO:152 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00226] SEQ ID NO:153 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00227] SEQ ID NO:154 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00228] SEQ ID NO:155 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00229] SEQ ID NO:156 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00230] SEQ ID NO:157 is the heavy chain amino acid sequence of the PD-L1 inhibitor avelumab.

[00231] SEQ ID NO:158 is the light chain amino acid sequence of the PD-L1 inhibitor avelumab.

[00232] SEQ ID NO:159 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor avelumab.

[00233] SEQ ID NO:160 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor avelumab.

[00234] SEQ ID NO:161 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

[00235] SEQ ID NO:162 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

[00236] SEQ ID NO:163 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

[00237] SEQ ID NO:164 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

[00238] SEQ ID NO:165 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor

avelumab.

[00239] SEQ ID NO:166 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

[00240] SEQ ID NO:167 is the heavy chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00241] SEQ ID NO:168 is the light chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00242] SEQ ID NO:169 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00243] SEQ ID NO:170 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00244] SEQ ID NO:171 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00245] SEQ ID NO:172 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00246] SEQ ID NO:173 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00247] SEQ ID NO:174 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00248] SEQ ID NO:175 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00249] SEQ ID NO:176 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

DETAILED DESCRIPTION OF THE INVENTION

[00250] 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.

Definitions

[00251] The term “frozen” means that the tissue, cellular composition, or other composition, has become hard or rigid as the result of exposure to cold. The term as used herein encompasses the concept of cryopreservation, which is a process of storing cells, tissue, etc., at very low temperatures wherein the stored cells, tissue, etc., maintain their viability.

[00252] The term “cryostorage” means the keeping of already frozen compositions at very low temperatures wherein the stored cells, tissue, etc., maintain their viability.

[00253] The terms “flash freezing,” “snap freezing,” “flash frozen,” and “snap frozen” mean causing to become frozen extremely rapidly, for non-limiting example, in less than about two minutes.

[00254] The term “LOVO cell processing system” and “LOVO” refers to the cell processing system manufactured by Fresenius Kabi USA, LLC. These two terms also refer 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, such a 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.

[00255] The term “fungin” refers to the antifungal reagent Fungin™ sold by InvitroGen, San Diego, CA, USA, (catalog numbers ant-fn-1 and ant-fn-2). Fungin is a soluble formulation of pimarin, CAS 7681-93-8. As used herein, “fungin” encompasses any commercial formulation of pimarin or natamycin.

[00256] The term “fungizone” is a trademark of E. R. Squibb and Sons, LLC, and refers to the antimycotic amphotericin B, CAS 1397-89-3. Amphotericin B is commercially available, for example from SIGMA-Aldrich, St. Louis, MO, USA, (catalog number A2942, as a 250 µg/mL solution in deionized water). As used herein, “fungizone” encompasses any commercial formulation of amphotericin B.

[00257] The term “physiologically buffered isotonic saline solution” means any one of the many such salt solutions known to the skilled artisan wherein the solution is made to a physiological

pH and isotonic salt concentration. In the art, these are commonly referred to as balanced salt solutions. Without limitation such physiologically buffered isotonic saline solution may comprise Hank's Balanced Salt Solution ("HBSS"), Tris-buffered saline ("TBS"), Phosphate buffered Saline ("PBS"), or Dulbecco's Phosphate Buffered Saline ("DPBS" or "dPBS").

[00258] The term "in vivo" refers to an event that takes place in a subject's body.

[00259] 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.

[00260] 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.

[00261] 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.

[00262] 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.

[00263] 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.

[00264] By “cryopreserved TILs” herein is meant that TILs, either primary, bulk, or expanded (REP TILs), are treated and stored at a temperature 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 and the avoidance of doubt, “cryopreserved TILs” are distinguishable from frozen tissue samples which may be used as a source of primary TILs.

[00265] 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.

[00266] 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 $\alpha\beta$, 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.

[00267] 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 5% v/v DMSO to 10% v/v DMSO; such media can also include media comprising 7% v/v DMSO to 10% v/v 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.

[00268] 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 secrete 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.

[00269] 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 secrete 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.

[00270] 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 fragment is added to the closed system, the system is not opened to the outside environment until the TILs are ready to be administered to the patient.

[00271] 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.

[00272] 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.

[00273] 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 CD3 ϵ . Other anti-CD3 antibodies include, for example, oteixizumab, teplizumab, and visilizumab.

[00274] The term “OKT-3” (also referred to herein as “OKT3”) refers to a monoclonal antibody or biosimilar or variant thereof, including human, humanized, chimeric, or murine antibodies, directed against the CD3 receptor in the T cell antigen receptor of mature T cells, and includes commercially-available forms such as OKT-3 (30 ng/mL, MACS GMP CD3 pure, Miltenyi Biotech, Inc., San Diego, CA, USA) and muromonab or variants, conservative amino acid substitutions, glycoforms, or biosimilars thereof. The amino acid sequences of the heavy and light chains of muromonab are given in Table 1 (SEQ ID NO:1 and SEQ ID NO:2). A hybridoma capable of producing OKT-3 is deposited with the American Type Culture Collection and assigned the ATCC accession number CRL 8001. A hybridoma capable of producing OKT-3 is also deposited with European Collection of Authenticated Cell Cultures (ECACC) and assigned Catalogue No. 86022706.

TABLE 1. Amino acid sequences of muromonab.

Identifier	Sequence (One-Letter Amino Acid Symbols)							
SEQ ID NO:1 Muromonab heavy chain	QVQLQSGAE	LARPGASVKM	SCKASGYTFT	RYTMHWVKQR	PGQGLEWIGY	INPSRGYTNV		60
	NQKFQDKATL	TTDKSSSTAY	MQLSSTLTSED	SAVYYCARYY	DDHYCLDYWG	QGTTLTVSSA		120
	KTTAPSVYPL	APVCGGTGGS	SVTLGCLVKG	YFPEPVTLTW	NSGSLSSGVH	TTPAVLQSDL		180
	YTLSSSVTVT	SSTWPSQSIT	CNVAHPASST	KVDKKIEPRP	KSCDKTHTCP	PCPAPELLGG		240
	PSVFLFPPKP	KDTLMISRTT	EVTCCVVDVS	HEDPEVKFNW	YVDGVEVHNA	KTKPREEQYN		300
	STYRVVSVLT	VLHQDWLNGK	EYKCKVSNKA	LPAPIEKTIS	KAKGQPREPQ	VYTLPPSRDE		360
	LTKNQVSLTC	LVKGFYPSDI	AVEWESNGQP	ENNYKTTTPV	LDSDGSFFLY	SKLTVDKSRW		420
	QQGNVFSCSV	MHEALHNHYT	QKSLSLSPGK					450
SEQ ID NO:2 Muromonab light chain	QIVLTQSPAI	MSASPGEKVT	MTCSASSSVS	YMNWYQQKSG	TSPKRWIYDT	SKLASGVPAAH		60
	FRGSGSGTSY	SLTISGMEAE	DAATYYCQQW	SSNPFTFGSG	TKLEINRADT	APTVSIFPPS		120
	SEQLTSGGAS	VVCFLNNFYP	KDINVVKWID	GSERQNGVLN	SWTDQDSKDS	TYSMSSTLT		180
	TKDEYERHNS	YTCEATHKTS	TSPIVKSENR	NEC				213

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	DLQMLNGIN	NYKNPKLTRM	LTFKFYMPKK	ATELKHLCQL		60
	EEELKPLEEV	LNLAQSKNFH	LRPRDLISNI	NVIVLELKGS	ETTFMCEYAD	ETATIVEFLN		120
	RWITFCQSII	STLT						134
SEQ ID NO:4 Aldelesleukin	PTSSSTKKTQ	LQLEHLLDL	QMILNGINNY	KNPKLTRMLT	FKFYMPKKAT	ELKHLQCLEE		60
	ELKPLEEVLN	LAQSKNFHLR	PRDLISNINV	IVLELKGSST	TFMCEYADET	ATIVEFLNRW		120
SEQ ID NO:5 recombinant human IL-4 (rhIL-4)	ITFSQSIIST	LT						132
	MHKCDITLQE	IIKTLNLSLE	QKTLCTELTV	TDIFAASKNT	TEKETFCRAA	TVLRQFYSHH		60
	EKDTRCLGAT	AQQFHRHKQL	IRFLKRLDRN	LGWLAGLNSC	PVKEANQSTL	ENFLERLKTI		120
SEQ ID NO:6 recombinant human IL-7 (rhIL-7)	MREKYSKCSS							130
	MDCDIEGKDG	KQYESVLMVS	IDQLDLSMKE	IGSNCLNNEF	NFFKRHICDA	NKEGMFLFRA		60
	ARKLRQFLKM	NSTGDFDLHL	LKVSEGTTL	LNCTGQVKGR	KPAALGEAQP	TKSLEENKSL		120
SEQ ID NO:7	KEQKLNLDLC	FLKRLLEIK	TCWNKILMGT	KEH				153
	MNVVNVISDL	KKIEDLIQSM	HIDATLYTES	DVHPCKVTA	MKCFLEELQV	ISLESGDASI		60

recombinant human IL-15 (rhIL-15)	HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS	115
SEQ ID NO:8	MQDRHMIRMRLQLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG	60
recombinant human IL-21 (rhIL-21)	NNERIINVSIIKKLRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLQKMIHQ	120
	HLSSRTHGSE DS	132

[00275] 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 A1, 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 4,902,502, 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.

[00276] 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 IgG1 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).

[00277] 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).

[00278] 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).

[00279] 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.*, 13:379-95 (2014), 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).

[00280] 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, in vivo, and ex 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, the manner of administration, *etc.* which can readily be determined by one of ordinary skill in the art. 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.

[00281] A “therapeutic effect” as that term is used herein, encompasses a therapeutic benefit and/or a prophylactic benefit. A prophylactic effect includes delaying or eliminating the appearance of a disease or condition, delaying or eliminating the onset of symptoms of a disease

or condition, slowing, halting, or reversing the progression of a disease or condition, or any combination thereof.

[00282] “Pharmaceutically acceptable carrier” or “pharmaceutically acceptable excipient” is 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.

[00283] When ranges are used herein to describe, for example, physical or chemical properties such as molecular weight or chemical formulae, all combinations and subcombinations of ranges and specific embodiments therein are intended to be included. Use of the term "about" when referring to a number or a numerical range means that the number or numerical range referred to is an approximation within experimental variability (or within statistical experimental error), and thus the number or numerical range may vary. The variation is typically from 0% to 15%, preferably from 0% to 10%, more preferably from 0% to 5% of the stated number or numerical range. The term “comprising” (and related terms such as “comprise” or “comprises” or “having” or “including”) includes those embodiments such as, for example, an embodiment of any composition of matter, method or process that “consist of” or “consist essentially of” the described features.

[00284] Compounds of the invention also include antibodies. The terms “antibody” and its plural form “antibodies” refer to whole immunoglobulins and any antigen-binding fragment (“antigen-binding portion”) or single chains thereof. An “antibody” further refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen-binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain

constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions of an antibody may be further subdivided into regions of hypervariability, which are referred to as complementarity determining regions (CDR) or hypervariable regions (HVR), and which can be interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen epitope or epitopes. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (C1q) of the classical complement system.

[00285] The terms “antigen-binding portion” or “antigen-binding fragment” of an antibody (or simply “antibody portion”), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, IL-33, ST2, CD20, PD-1, PD-L1, or PD-L2). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term “antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment (Ward *et al.*, *Nature*, **1989**, 341, 544-546), which may consist of a V_H or a V_L domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules known as single chain Fv (scFv); see, *e.g.*, Bird *et al.*, *Science* **1988**, 242, 423-426; and Huston *et al.*, *Proc. Natl. Acad. Sci. USA* **1988**, 85, 5879-5883). Such scFv antibodies are also intended to be encompassed within the terms “antigen-binding portion” or “antigen-binding fragment” of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

[00286] The term “human antibody,” as used herein, is intended to include antibodies having

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

[00287] The term “human monoclonal antibody” refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences. In one embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

[00288] The term “recombinant human antibody”, as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[00289] As used herein, “isotype” refers to the antibody class (*e.g.*, IgM or IgG1) that is

encoded by the heavy chain constant region genes. In mammals, there are five antibody isotypes: IgA, IgD, IgG, IgM and IgE. In humans, there are four subclasses of the IgG isotype: IgG1, IgG2, IgG3 and IgG4, and two subclasses of the IgA isotype: IgA1 and IgA2.

[00290] The phrases “an antibody recognizing an antigen” and “an antibody specific for an antigen” are used interchangeably herein with the term “an antibody which binds specifically to an antigen.”

[00291] The term “human antibody derivatives” refers to any modified form of the human antibody, *e.g.*, a conjugate of the antibody and another active pharmaceutical ingredient or antibody. The terms “conjugate,” “antibody-drug conjugate,” “ADC,” or “immunoconjugate” refers to an antibody, or a fragment thereof, conjugated to a therapeutic moiety, such as a bacterial toxin, a cytotoxic drug or a radionuclide-containing toxin. Toxic moieties can be conjugated to antibodies of the invention using methods available in the art.

[00292] The terms “humanized antibody,” “humanized antibodies,” and “humanized” are intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences. Humanized forms of non-human (for example, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a 15 hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc),

typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature* **1986**, 321, 522-525; Riechmann *et al.*, *Nature* **1988**, 332, 323-329; and Presta, *Curr. Op. Struct. Biol.* **1992**, 2, 593-596.

[00293] The term “chimeric antibody” is intended to refer to antibodies in which the variable region sequences are derived from one species and the constant region sequences are derived from another species, such as an antibody in which the variable region sequences are derived from a mouse antibody and the constant region sequences are derived from a human antibody.

[00294] A “diabody” is a small antibody fragment with two antigen-binding sites. The fragments comprises a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L or V_L-V_H). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, *e.g.*, European Patent No. EP 404,097, International Patent Publication No. WO 93/11161; and Bolliger *et al.*, *Proc. Natl. Acad. Sci. USA* **1993**, 90, 6444-6448.

[00295] The term “glycosylation” refers to a modified derivative of an antibody. An aglycosylated antibody lacks glycosylation. Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Aglycosylation may increase the affinity of the antibody for antigen, as described in U.S. Patent Nos. 5,714,350 and 6,350,861. Additionally, or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. For example, the cell lines Ms704, Ms705, and Ms709 lack the fucosyltransferase gene, FUT8 (alpha

(1,6) fucosyltransferase), such that antibodies expressed in the Ms704, Ms705, and Ms709 cell lines lack fucose on their carbohydrates. The Ms704, Ms705, and Ms709 FUT8^{-/-} cell lines were created by the targeted disruption of the FUT8 gene in CHO/DG44 cells using two replacement vectors (*see e.g.* U.S. Patent Publication No. 2004/0110704 or Yamane-Ohnuki, *et al. Biotechnol. Bioeng.*, **2004**, 87, 614-622). As another example, European Patent No. EP 1,176,195 describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation by reducing or eliminating the alpha 1,6 bond-related enzyme, and also describes cell lines which have a low enzyme activity for adding fucose to the N-acetylglucosamine that binds to the Fc region of the antibody or does not have the enzyme activity, for example the rat myeloma cell line YB2/0 (ATCC CRL 1662). International Patent Publication WO 03/035835 describes a variant CHO cell line, Lec 13 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (*see also* Shields, *et al.*, *J. Biol. Chem.* **2002**, 277, 26733-26740. International Patent Publication WO 99/54342 describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.*, beta(1,4)-N-acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNAc structures which results in increased ADCC activity of the antibodies (*see also* Umana, *et al.*, *Nat. Biotech.* **1999**, 17, 176-180). Alternatively, the fucose residues of the antibody may be cleaved off using a fucosidase enzyme. For example, the fucosidase alpha-L-fucosidase removes fucosyl residues from antibodies as described in Tarentino, *et al.*, *Biochem.* **1975**, 14, 5516-5523.

[00296] “Pegylation” refers to a modified antibody, or a fragment thereof, that typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Pegylation may, for example, increase the biological (*e.g.*, serum) half life of the antibody. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term “polyethylene glycol” is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C₁-C₁₀) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. The antibody to be pegylated may be an aglycosylated antibody. Methods for pegylation are known in the art and can be applied to the

antibodies of the invention, as described for example in European Patent Nos. EP 0154316 and EP 0401384.

[00297] The term “biosimilar” means a biological product that is highly similar to a U.S. licensed reference biological product notwithstanding minor differences in clinically inactive components, and for which there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product. Furthermore, a similar biological or “biosimilar” medicine is a biological medicine that is similar to another biological medicine that has already been authorized for use by the European Medicines Agency. The term “biosimilar” is also used synonymously by other national and regional regulatory agencies. Biological products or biological medicines are medicines that are made by or derived from a biological source, such as a bacterium or yeast. They can consist of relatively small molecules such as human insulin or erythropoietin, or complex molecules such as monoclonal antibodies. For example, if the reference anti-CD20 monoclonal antibody is rituximab, an anti-CD20 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to rituximab is a “biosimilar to” rituximab or is a “biosimilar thereof” of rituximab. In Europe, a similar biological or “biosimilar” medicine is a biological medicine that is similar to another biological medicine that has already been authorized for use by the European Medicines Agency (EMA). The relevant legal basis for similar biological applications in Europe is Article 6 of Regulation (EC) No 726/2004 and Article 10(4) of Directive 2001/83/EC, as amended and therefore in Europe, the biosimilar may be authorised, approved for authorisation or subject of an application for authorisation under Article 6 of Regulation (EC) No 726/2004 and Article 10(4) of Directive 2001/83/EC. The already authorized original biological medicinal product may be referred to as a “reference medicinal product” in Europe. Some of the requirements for a product to be considered a biosimilar are outlined in the CHMP Guideline on Similar Biological Medicinal Products. In addition, product specific guidelines, including guidelines relating to monoclonal antibody biosimilars, are provided on a product-by-product basis by the EMA and published on its website. A biosimilar as described herein may be similar to the reference medicinal product by way of quality characteristics, biological activity, mechanism of action, safety profiles and/or efficacy. In addition, the biosimilar may be used or be intended for use to treat the same conditions as the reference medicinal product. Thus, a biosimilar as described herein may be deemed to have similar or highly similar quality

characteristics to a reference medicinal product. Alternatively, or in addition, a biosimilar as described herein may be deemed to have similar or highly similar biological activity to a reference medicinal product. Alternatively, or in addition, a biosimilar as described herein may be deemed to have a similar or highly similar safety profile to a reference medicinal product. Alternatively, or in addition, a biosimilar as described herein may be deemed to have similar or highly similar efficacy to a reference medicinal product. As described herein, a biosimilar in Europe is compared to a reference medicinal product which has been authorised by the EMA. However, in some instances, the biosimilar may be compared to a biological medicinal product which has been authorised outside the European Economic Area (a non-EEA authorised “comparator”) in certain studies. Such studies include for example certain clinical and in vivo non-clinical studies. As used herein, the term “biosimilar” also relates to a biological medicinal product which has been or may be compared to a non-EEA authorised comparator. Certain biosimilars are proteins such as antibodies, antibody fragments (for example, antigen binding portions) and fusion proteins. A protein biosimilar may have an amino acid sequence that has minor modifications in the amino acid structure (including for example deletions, additions, and/or substitutions of amino acids) which do not significantly affect the function of the polypeptide. The biosimilar may comprise an amino acid sequence having a sequence identity of 97% or greater to the amino acid sequence of its reference medicinal product, *e.g.*, 97%, 98%, 99% or 100%. The biosimilar may comprise one or more post-translational modifications, for example, although not limited to, glycosylation, oxidation, deamidation, and/or truncation which is/are different to the post-translational modifications of the reference medicinal product, provided that the differences do not result in a change in safety and/or efficacy of the medicinal product. The biosimilar may have an identical or different glycosylation pattern to the reference medicinal product. Particularly, although not exclusively, the biosimilar may have a different glycosylation pattern if the differences address or are intended to address safety concerns associated with the reference medicinal product. Additionally, the biosimilar may deviate from the reference medicinal product in for example its strength, pharmaceutical form, formulation, excipients and/or presentation, providing safety and efficacy of the medicinal product is not compromised. The biosimilar may comprise differences in for example pharmacokinetic (PK) and/or pharmacodynamic (PD) profiles as compared to the reference medicinal product but is still deemed sufficiently similar to the reference medicinal product as to be authorised or

considered suitable for authorisation. In certain circumstances, the biosimilar exhibits different binding characteristics as compared to the reference medicinal product, wherein the different binding characteristics are considered by a Regulatory Authority such as the EMA not to be a barrier for authorisation as a similar biological product. The term “biosimilar” is also used synonymously by other national and regional regulatory agencies.

[00298] 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, ALL, CLL, 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.

[00299] 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.

[00300] 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).

[00301] 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.

[00302] 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.

[00303] 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

[00304] The terms “sequence identity,” “percent identity,” and “sequence percent identity” 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.

[00305] Certain embodiments of the present invention comprise a variant of an antibody, *e.g.*, an anti-IL-33 or anti-ST2 antibody and/or an anti-CD20 antibody and/or an anti-PD-1 antibody, anti-PD-L1 and/or an anti-PD-L2 antibody. As used herein, the term “variant” encompasses but is not limited to antibodies 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.

[00306] For the avoidance of doubt, it is intended herein that particular features (for example integers, characteristics, values, uses, diseases, formulae, compounds or groups) described in conjunction with a particular aspect, embodiment or example of the invention are to be understood as applicable to any other aspect, embodiment or example described herein unless incompatible therewith. Thus such features may be used where appropriate in conjunction with any of the definition, claims or embodiments defined herein. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of the features and/or steps are mutually exclusive. The invention is not restricted to any details of any disclosed embodiments. The invention extends to any novel one, or novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

Tumor Cryopreservation Methods

[00307] The invention provides a method for cryopreserving tumor tissue for the manufacture of tumor infiltrating lymphocytes (TILs) comprising:

- (i) fragmenting the tumor tissue;
- (ii) incubating the fragments in a cryopreservation medium; and,
- (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

[00308] In an embodiment, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to about 6 mm. In a preferred embodiment, the approximately spherical fragments have a diameter of about 6 mm. In an embodiment, the approximately spherical fragments have a diameter of about 3 mm.

[00309] In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 1.5 mm and a longest edge length of about 6 mm. In an embodiment, the tumor tissue is fragmented into generally cubical fragments having edge lengths of between about 1.5 mm and 6 mm. In an embodiment, the generally cubical fragments have edge lengths of about 6 mm. In an embodiment, the generally cubical fragments have edge lengths of about 3 mm.

[00310] In some embodiments, the tissue sample is trimmed to separate non-tumor tissue from tumor tissue.

[00311] In some embodiments, the tumor tissue is from a dissected tumor. In an embodiment, the tumor tissue is from a tumor biopsy. In some embodiments the tumor tissue is from an incisional biopsy. In some embodiments the tumor tissue is from an excisional biopsy. In some embodiments the tumor tissues may be from one or more core needle biopsies.

[00312] In some embodiments, the fresh tumor tissue is trimmed into fragments with a cross section of about 1.5 mm × 1.5 mm, about 2 mm × 2 mm, about 2.5 mm × 2.5 mm, about 3 mm × 3 mm, about 3.5 mm × 3.5 mm, about 4 mm × 4 mm, about 4.5 mm × 4.5 mm, about 5 mm × 5 mm, about 5.5 mm × about 5.5 mm, or about 6 mm × about 6 mm.

[00313] In an embodiment, the tumor tissue is less than twelve hours old. In an embodiment, the tumor tissue is less than eight hours old. In an embodiment, the tumor tissue is less than three, less than two, or less than one-hour old.

[00314] In some embodiments, the cryopreservation medium comprises 2 to 12 % v/v (volume:volume) dimethylsulfoxide (DMSO). In some embodiments the cryopreservation medium comprises 5% v/v DMSO. In some embodiments the cryopreservation medium comprises 10% v/v DMSO. In some embodiments, the cryopreservation medium comprises between 5 and 10 % v/v DMSO. In some embodiments the cryopreservation medium comprises a percentage of DMSO selected from the group consisting of 1% v/v, 2% v/v, 3% v/v, 4% v/v, 5% v/v, 6% v/v, 7% v/v, 8% v/v, 9% v/v, 10% v/v, 11% v/v, 12% v/v, 13% v/v, 14% v/v, and 15% v/v. In some of the foregoing embodiments, the remainder of the cryopreservation medium is an aqueous medium.

[00315] In some embodiments, the cryopreservation medium comprises 2 to 12 % w/w (weight:weight) dimethylsulfoxide (DMSO). In some embodiments the cryopreservation medium comprises 5% w/w DMSO. In some embodiments the cryopreservation medium comprises 10% w/w DMSO. In some embodiments, the cryopreservation medium comprises between 5 and 10 % w/w DMSO. In some embodiments the cryopreservation medium comprises a percentage of DMSO selected from the group consisting of 1% w/w, 2% w/w, 3% w/w, 4% w/w, 5% w/w, 6% w/w, 7% w/w, 8% w/w, 9% w/w, 10% w/w, 11% w/w, 12% w/w, 13% w/w, 14% w/w, and 15% w/w. In some of the foregoing embodiments, the remainder of the cryopreservation medium is an aqueous medium.

[00316] In an embodiment, the cryopreservation medium comprises at least one antimicrobial agent. In an embodiment, the at least one antimicrobial agent is gentamicin at a concentration of at least 50 µg/mL. In some embodiments, the at least one antimicrobial agent is penicillin; in some embodiments the at least one antimicrobial agent is streptomycin. In some embodiments the at least one antimicrobial agent is an antifungal agent. In some embodiments the antifungal agent is amphotericin B; in some embodiments the antifungal agent is Fungin™. In some embodiments combinations of antimicrobial agents are used. In some embodiments at least one antifungal agent is used in combination with one or more antibacterial agents.

[00317] In some embodiments, the tumor fragments are incubated in cryopreservation medium for about 20 minutes to about 70 minutes. In some embodiments, the tumor fragments are incubated in cryopreservation medium for about 30 minutes to about 60 minutes. In some embodiments the incubation is at least 10 minutes; at least 20 minutes; at least 25 minutes; at least 30 minutes; at least 35 minutes; at least 40 minutes; at least 45 minutes; at least 50 minutes; at least 55 minutes; at least 60 minutes; or at least 70 minutes. In some embodiments the incubation is about 10 minutes; about 20 minutes; about 25 minutes; about 30 minutes; about 35 minutes; about 40 minutes; about 45 minutes; about 50 minutes; about 55 minutes; about 60 minutes; or about 70 minutes. In some embodiments the incubation is less than 10 minutes; less than 20 minutes; less than 25 minutes; less than 30 minutes; less than 35 minutes; less than 40 minutes; less than 45 minutes; less than 50 minutes; less than 55 minutes; less than 60 minutes; or less than 70 minutes. In some embodiments the incubation time is proportional to tumor fragment density. In some embodiments the incubation time is proportional to tumor fragment surface to volume ratio.

[00318] In some embodiments, the tumor fragments are incubated in a cryopreservation medium at a temperature from about 2°C to about 8°C.

[00319] In some embodiments, the tumor tissue is washed in a physiologically buffered isotonic saline solution. In some embodiments, the washing comprises three serial washes of at least three minutes each, with the physiologically buffered isotonic saline solution replaced after each serial wash. In some embodiments, the physiologically buffered isotonic saline solution comprises Hank's Balance Salt Solution (HBSS). In some embodiments, the physiologically buffered isotonic saline solution comprises tris-buffered saline (TBS). In some embodiments, the physiologically buffered isotonic saline solution comprises phosphate buffered saline (PBS). In some embodiments, the physiologically buffered isotonic saline solution comprises Dulbecco's phosphate-buffered saline (DPBS). In some embodiments, the physiologically buffered isotonic saline solution in one serial wash may be a different physiologically buffered isotonic saline solution than used in one or more of the other serial washes.

[00320] In an embodiment, the freezing takes place at a temperature in the range from about -125°C to about -196°C. In an embodiment, the freezing takes place at a temperature in the range of about -140°C to about -185°C. In an embodiment, the freezing takes place at a

temperature in the range of about -140°C to about -175°C. In an embodiment, the freezing takes place at a temperature of about -145°C. In some embodiments, the freezing takes place in the vapor phase of liquid nitrogen.

[00321] A problem well-known in the art is to cryopreserve cells or tissues without damaging them during the freezing process. Without being bound by theory, one source of damage during freezing is intracellular ice nucleation resulting in cellular rupture. Muldrew and McGann outline a quantitative theory for this well known and widely recognized difficulty with cellular and, in particular, whole tissue cryopreservation in “The osmotic rupture hypothesis of intracellular freezing injury”, *Biophysical Journal*, **66**:532-41 (1994). Acker and McGann further develop the fundamental mechanisms of intracellular ice formation and cellular damage in a later article, “Membrane damage occurs during the formation of intracellular ice,” *Cryo Letter*, **22**:241-54 (2001).

[00322] Damage during freezing is detected wherein upon thawing, the tissues have substantially lost their physiological structures or the cells comprising the tissue have substantially lost their viability. Viability may be determined by the fraction of cells introduced into a culture medium compared to the number of such cells that grow or show markers of normal cellular function. Numerous methods are known in the art to identify the fraction of viable cells, for example, and without limitation, dye exclusion tests, such as trypan blue exclusion. See, for example, Strober, *Curr. Protoc. Immunol.*, **2001**, Appendix 3B, available at <https://dx.doi.org/10.1002/0471142735.ima03bs21>. Without limitation, viability may also be determined by metabolic activity assays, such as the MTT assay, wherein MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is metabolized by cellular enzymes into formazan. This enzymatic reaction converts the yellow MTT into purple formazan. See, for example, Berridge *et al.*, Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology Annual Review*, **11**: 127-152 (2005); Mosmann, “Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays,” *J. Immunol. Methods* **65** (1–2): 55–63 (1983).

[00323] Without being bound by theory, slow cooling is hypothesized to produce innocuous intracellular ice, see Acker and McGann, “Protective effect of intracellular ice during freezing?” *Cryobiology*, **46**(2): 197-202 (2003). A conventional approach to achieve freezing without

excessive cellular damage or to achieve freezing without significantly reducing cellular viability has been to employ slow freezing rates. Watson *et al.*, U.S. Patent 5,891,617, emphasize this approach, for example claim one, step (c), teaches a very slow rate of cooling: “about -0.3° C. per minute or less.” Similarly, Comhaire *et al.*, U.S. Patent 9,938,495, teach “high cell viability after thawing were obtained by slow freezing using a DMSO-free cryopreservation medium” for stem cells.

[00324] Based on these exemplary teachings, the methods and products of the present disclosure are surprising and unexpected. Further the present disclosure, including the examples and the data therein, demonstrate a technical solution to the problem of rapidly and efficiently cryoprotecting tumor tissues, tumor fragments, or tumor specimens, for the use in the manufacture of tumor infiltrating lymphocytes for therapeutic use.

[00325] In some embodiments, the method of cryopreserving tumor tissue for the manufacture of tumor infiltrating lymphocytes (TILs), further comprises a step (iv) storing the frozen fragments at a temperature below at least -130°C. In some embodiments, the frozen fragments are stored in the vapor phase of liquid nitrogen. In some embodiments, the frozen fragments are stored submerged in liquid nitrogen. In some embodiments, the cryopreserved fragments are stored for later manufacture of TILs for autologous therapeutic use.

[00326] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising the steps of:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising incubating the tumor tissue for a period of about 20 minutes to about 70 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing;
- (b) thawing the tumor tissue;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;
- (d) removing at least a plurality of the TILs; and
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to

provide an expanded number of TILs.

[00327] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising the steps of:

- (a) fragmenting a tumor sample;
- (b) incubating the tumor fragments in a storage medium;
- (c) storing the tumor fragments in a frozen state;
- (d) thawing the tumor fragments;
- (e) treating the tumor fragments in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;
- (f) removing at least a plurality of the TILs; and
- (g) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00328] In some embodiments, the storage medium comprises 2 to 12 % v/v (volume:volume or volume-to-volume) dimethylsulfoxide (DMSO). In some embodiments the storage medium comprises 5% v/v DMSO. In some embodiments the storage medium comprises 10% v/v DMSO. In some embodiments, the storage medium comprises between 5 and 10 % v/v DMSO. In some embodiments the storage medium comprises a percentage of DMSO selected from the group consisting of 1% v/v, 2% v/v, 3% v/v, 4% v/v, 5% v/v, 6% v/v, 7% v/v, 8% v/v, 9% v/v, 10% v/v, 11% v/v, 12% v/v, 13% v/v, 14% v/v, and 15% v/v. In some of the foregoing embodiments, the remainder of the storage medium is an aqueous medium.

[00329] In some embodiments, the storage medium comprises 2 to 12 % w/w (weight:weight or weight-to-weight) dimethylsulfoxide (DMSO). In some embodiments the storage medium comprises 5% w/w DMSO. In some embodiments the storage medium comprises 10% w/w DMSO. In some embodiments, the storage medium comprises between 5 and 10 % w/w DMSO. In some embodiments the storage medium comprises a percentage of DMSO selected from the group consisting of 1% w/w, 2% w/w, 3% w/w, 4% w/w, 5% w/w, 6% w/w, 7% w/w, 8% w/w,

9% w/w, 10% w/w, 11% w/w, 12% w/w, 13% w/w, 14% w/w, and 15% w/w. In some of the foregoing embodiments, the remainder of the storage medium is an aqueous medium.

[00330] In some embodiments, the tumor tissue is incubated for between about 20 minutes and about 70 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for at least 30 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for between 30 minutes and about 60 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 15 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 45 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 75 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for 60 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing.

[00331] In some embodiments, the storage medium comprises about 5% v/v DMSO and the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing. In some embodiments, the storage medium comprises about 10% v/v DMSO and the tumor tissue is incubated for at least 30 minutes to about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments the incubation time is proportional to tumor fragment density. In some embodiments the incubation time is proportional to tumor fragment surface to volume ratio.

Cryopreserved Tumor Fragments

[00332] In some embodiments, the invention provides a cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by a process comprising the steps of:

- (i) fragmenting a tumor sample;
- (ii) incubating the fragments in a cryopreservation medium; and,
- (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

[00333] In some embodiments, the invention provides a cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by a process comprising the steps of:

- (i) fragmenting a tumor sample;
- (ii) incubating the fragments for about 20 minutes to about 70 minutes at a temperature in the range of about 2°C to about 8°C in a cryopreservation medium comprising 10% v/v DMSO; and,
- (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

[00334] In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 6 mm. In an embodiment, the approximately spherical fragments have a diameter of about 6 mm. In an embodiment, the approximately spherical fragments have a diameter of about 3 mm. In an embodiment, the approximately spherical fragments have a diameter selected from the group consisting of about 20 mm, about 19 mm, about 18 mm, about 17 mm, about 16 mm, about 15 mm, about 14 mm, about 13 mm, about 12 mm, about 11 mm, about 10 mm, about 9 mm, about 8 mm, about 7 mm, about 6 mm, about 5 mm, about 4 mm, about 3 mm, about 2 mm, and about 1 mm.

[00335] In some embodiments, the tumor sample is fragmented into generally rectangular fragments having a shortest edge length of at least 1.5 mm and a longest edge of about 6 mm. In an embodiment, the tumor sample is fragmented into generally cubical fragments having edges with lengths of between about 1.5 mm and 6 mm. In an embodiment, the generally cubical fragments have edges with lengths of about 6 mm. In an embodiment, the generally cubical

fragments have edges with lengths of about 3 mm. In an embodiment, the generally cubical fragments have an edge length selected from the group consisting of about 20 mm, about 19 mm, about 18 mm, about 17 mm, about 16 mm, about 15 mm, about 14 mm, about 13 mm, about 12 mm, about 11 mm, about 10 mm, about 9 mm, about 8 mm, about 7 mm, about 6 mm, about 5 mm, about 4 mm, about 3 mm, about 2 mm, and about 1 mm.

[00336] In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 2 mm^3 to about 200 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 5 mm^3 to about 150 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 25 mm^3 to about 150 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 50 mm^3 to about 150 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 100 mm^3 to about 125 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 50 mm^3 to about 75 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 75 mm^3 to about 100 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 200 mm^3 to about 1000 mm^3 . In an embodiment, the tumor sample is fragmented into approximately spherical fragments having a volume of about 500 mm^3 to about 800 mm^3 . In an embodiment, the approximately spherical fragments have a diameter selected from the group consisting of about 20 mm, about 19 mm, about 18 mm, about 17 mm, about 16 mm, about 15 mm, about 14 mm, about 13 mm, about 12 mm, about 11 mm, about 10 mm, about 9 mm, about 8 mm, about 7 mm, about 6 mm, about 5 mm, about 4 mm, about 3 mm, about 2 mm, and about 1 mm.

[00337] In some embodiments, the fresh tumor tissue is trimmed into fragments, wherein a fragment has a cross section of about $1.5 \text{ mm} \times 1.5 \text{ mm}$, about $2 \text{ mm} \times 2 \text{ mm}$, about $2.5 \text{ mm} \times 2.5 \text{ mm}$, about $3 \text{ mm} \times 3 \text{ mm}$, about $3.5 \text{ mm} \times 3.5 \text{ mm}$, about $4 \text{ mm} \times 4 \text{ mm}$, about $4.5 \text{ mm} \times 4.5 \text{ mm}$, about $5 \text{ mm} \times 5 \text{ mm}$, about $5.5 \text{ mm} \times 5.5 \text{ mm}$, about $6 \text{ mm} \times 6 \text{ mm}$, about $6.5 \text{ mm} \times 6.5 \text{ mm}$, about $7 \text{ mm} \times 7 \text{ mm}$, about $7.5 \text{ mm} \times 7.5 \text{ mm}$, about $8 \text{ mm} \times 8 \text{ mm}$, about $8.5 \text{ mm} \times 8.5 \text{ mm}$, about $9 \text{ mm} \times 9 \text{ mm}$, about $9.5 \text{ mm} \times 9.5 \text{ mm}$, about $10 \text{ mm} \times 10 \text{ mm}$, about $10.5 \text{ mm} \times 10.5 \text{ mm}$, about $11 \text{ mm} \times 11 \text{ mm}$, about $11.5 \text{ mm} \times 11.5 \text{ mm}$, about $12 \text{ mm} \times 12 \text{ mm}$.

[00338] In some embodiments, the fresh tumor tissue is trimmed into fragments, wherein a fragment has a cross-sectional area of about 2 mm² to about 3 mm², about 3 mm² to about 4 mm², about 4 mm² to about 5 mm², about 5 mm² to about 6 mm², about 6 mm² to about 7 mm², about 7 mm² to about 8 mm², about 8 mm² to about 9 mm², about 9 mm² to about 10 mm², about 10 mm² to about 11 mm², about 11 mm² to about 12 mm², about 12 mm² to about 20 mm², about 20 mm² to about 50 mm², about 50 mm² to about 100 mm², or about 100 mm² to about 500 mm². In some embodiments, the fresh tumor tissue is trimmed into fragments, wherein a fragment has a cross-sectional area of about 1.5 mm², about 2 mm², about 2.5 mm², about 3 mm², about 3.5 mm², about 4 mm², about 4.5 mm², about 5 mm², about 5.5 mm², about 6 mm², about 6.5 mm², about 7 mm², about 7.5 mm², about 8 mm², about 8.5 mm², about 9 mm², about 9.5 mm², about 10 mm², about 10.5 mm², about 11 mm², about 11.5 mm², about 12 mm², about 20 mm², about 25 mm², about 30 mm², about 40 mm², about 50 mm², about 100 mm², about 200 mm², about 300 mm², about 400 mm², about 500 mm², about 750 mm², or about 1000 mm².

[00339] In some embodiments, the fresh tumor tissue is trimmed into fragments, wherein a fragment has a volume of about 2 mm³ to about 3 mm³, about 3 mm³ to about 4 mm³, about 4 mm³ to about 5 mm³, about 5 mm³ to about 6 mm³, about 6 mm³ to about 7 mm³, about 7 mm³ to about 8 mm³, about 8 mm³ to about 9 mm³, about 9 mm³ to about 10 mm³, about 10 mm³ to about 11 mm³, about 11 mm³ to about 12 mm³, and about 12 mm³ to about 20 mm³. In some embodiments, the fresh tumor tissue is trimmed into fragments, wherein a fragment has a volume of about 1.5 mm³, about 2 mm³, about 2.5 mm³, about 3 mm³, about 3.5 mm³, about 4 mm³, about 4.5 mm³, about 5 mm³, about 5.5 mm³, about 6 mm³, about 6.5 mm³, about 7 mm³, about 7.5 mm³, about 8 mm³, about 8.5 mm³, about 9 mm³, about 9.5 mm³, about 10 mm³, about 10.5 mm³, about 11 mm³, about 11.5 mm³, about 12 mm³, about 20 mm³, about 25 mm³, about 30 mm³, about 40 mm³, about 50 mm³, about 100 mm³, about 200 mm³, about 300 mm³, about 400 mm³, about 500 mm³, about 750 mm³, or about 1000 mm³.

[00340] In some embodiments, the tumor sample is from a dissected tumor. In an embodiment, the tumor sample is from a tumor biopsy. In some embodiments the tumor sample is from an incisional biopsy. In some embodiments the tumor sample is from an excisional biopsy. In some embodiments the tumor samples may be from one or more core needle biopsies.

[00341] In an embodiment, the tumor sample is less than twelve hours old prior to freezing. In an embodiment, the tumor sample is less than eight hours old prior to freezing. In an embodiment, the tumor sample is less than three, less than two, or less than one-hour old prior to freezing.

[00342] In some embodiments, the cryopreservation medium comprises 2 to 12 % v/v (volume:volume or volume-to-volume) dimethylsulfoxide (DMSO). In some embodiments the cryopreservation medium comprises 5% v/v DMSO. In some embodiments the cryopreservation medium comprises 10% v/v DMSO. In some embodiments, the cryopreservation medium comprises between 5 and 10 % v/v DMSO. In some embodiments the cryopreservation medium comprises a percentage of DMSO selected from the group consisting of 1% v/v, 2% v/v, 3% v/v , 4% v/v , 5% v/v , 6% v/v, 7% v/v, 8% v/v, 9% v/v, 10% v/v, 11% v/v, 12% v/v, 13% v/v, 14% v/v, and 15% v/v. In some of the foregoing embodiments, the remainder of the cryopreservation medium is an aqueous medium.

[00343] In some embodiments, the cryopreservation medium comprises 2 to 12 % w/w (weight:weight or weight-to-weight) dimethylsulfoxide (DMSO). In some embodiments the cryopreservation medium comprises 5% w/w DMSO. In some embodiments the storage medium comprises 10% w/w DMSO. In some embodiments, the cryopreservation medium comprises between 5 and 10 % w/w DMSO. In some embodiments the cryopreservation medium comprises a percentage of DMSO selected from the group consisting of 1% w/w, 2% w/w, 3% w/w , 4% w/w , 5% w/w , 6% w/w, 7% w/w, 8% w/w, 9% w/w, 10% w/w, 11% w/w, 12% w/w, 13% w/w, 14% w/w, and 15% w/w. In some of the foregoing embodiments, the remainder of the cryopreservation medium is an aqueous medium.

[00344] In an embodiment, the cryopreservation medium comprises at least one antimicrobial agent. In an embodiment, the at least one antimicrobial agent is gentamicin at a concentration of at least 20 µg/mL. In some embodiments, the at least one antimicrobial agent is gentamicin at a concentration of at least 50 µg/mL. In some embodiments, the at least one antimicrobial agent is penicillin; in some embodiments the at least one antimicrobial agent is streptomycin. In some embodiments the at least one antimicrobial agent is an antifungal agent. In some embodiments the antifungal agent is amphotericin B; in some embodiments the antifungal agent is Fungin™.

In some embodiments combinations of antimicrobial agents are used. In some embodiments at least one antifungal agent is used in combination with one or more antibacterial agents.

[00345] In some embodiments, the tumor fragments are incubated in cryopreservation medium for about 30 minutes to about 60 minutes. In some embodiments the incubation is at least 10 minutes; at least 20 minutes; at least 25 minutes; at least 30 minutes; about 35 minutes; about 40 minutes; about 45 minutes; about 50 minutes; about 55 minutes; or about 60 minutes. In some embodiments the incubation time is proportional to tumor fragment density. In some embodiments the incubation time is proportional to tumor fragment surface to volume ratio.

[00346] In some embodiments, the tumor fragments are incubated in cryopreservation medium at a temperature from about 2°C to about 8°C.

[00347] In some embodiments, the tumor sample is washed in a physiologically buffered isotonic saline solution. In some embodiments, the washing comprises three serial washes of at least three minutes each, with the physiologically buffered isotonic saline solution replaced after each serial wash. In some embodiments, the physiologically buffered isotonic saline solution comprises Hank's Balance Salt Solution (HBSS). In some embodiments, the physiologically buffered isotonic saline solution comprises tris-buffered saline (TBS). In some embodiments, the physiologically buffered isotonic saline solution comprises phosphate buffered saline (PBS). In some embodiments, the physiologically buffered isotonic saline solution comprises Dulbecco's phosphate-buffered saline (DPBS). In some embodiments, the physiologically buffered isotonic saline solution in one serial wash may be a different physiologically buffered isotonic saline solution than used in one or more of the other serial washes.

[00348] In an embodiment, the freezing takes place at a temperature in the range from about -125°C to about -180°C. In an embodiment, the freezing takes place at a temperature in the range of about -140°C to about -175°C. In an embodiment, the freezing takes place at a temperature of about -145°C. In some embodiments, the freezing takes place in the vapor phase of liquid nitrogen.

[00349] In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is one. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is two. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is three. In an

embodiment, the number of tumor fragments used according to any of the embodiments above or herein is four. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is five. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is six. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is seven. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is eight. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is nine. In an embodiment, the number of tumor fragments used according to any of the embodiments above or herein is ten.

Manufacturing TILs from Cryopreserved Tumor Fragments

[00350] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from a cryopreserved tumor fragment into a therapeutic population of TILs, the method comprising the following steps:

- (i) obtaining a first population of TILs from a cryopreserved tumor fragment;
- (ii) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and optionally OKT-3 to produce a second population of TILs; and
- (iii) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the third population of TILs is at least 50-fold or 100-fold greater in number than the second population of TILs, and wherein the second expansion is performed for at least 14 days in order to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs.

[00351] The invention provides a method for preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy, the method comprising:

(a) culturing a cryopreserved tumor fragment in a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;

(b) expanding the TILs in a second cell culture medium comprising irradiated feeder cells, OKT-3 antibody, and IL-2 to provide an expanded number of TILs; and,

(c) optionally cryopreserving the expanded number of TILs.

[00352] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising the steps of:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising incubating the tumor tissue for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing;
- (b) thawing the tumor tissue;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (d) removing at least a plurality of the TILs; and
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00353] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising the steps of:

- (a) fragmenting a tumor sample;
- (b) incubating the tumor fragments in a storage medium for about 30 minutes at a temperature in the range of about 2°C to about 8°C;
- (c) storing the tumor fragments in a frozen state;
- (d) thawing the tumor fragments;
- (e) treating the tumor fragments in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;

- (f) removing at least a plurality of the TILs; and
- (g) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00354] In some embodiments, the storage medium comprises 2% v/v DMSO to 12 % v/v dimethylsulfonoxide (DMSO). In some embodiments the storage medium comprises 5% v/v DMSO. In some embodiments the storage medium comprises 10% v/v DMSO. In some embodiments, the storage medium comprises between about 5% v/v DMSO and 10 % v/v DMSO.

[00355] In some embodiments, the tumor tissue is incubated for at least 30 minutes in a storage medium at a temperature from about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for between 30 minutes and about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 45 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing. In some embodiments, the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C before freezing.

[00356] In some embodiments, the tumor tissue is incubated in cryopreservation medium for about 20 minutes to about 70 minutes. In some embodiments, the tumor tissue is incubated in cryopreservation medium for about 30 minutes to about 60 minutes. In some embodiments the incubation is at least 10 minutes; at least 20 minutes; at least 25 minutes; at least 30 minutes; about 35 minutes; about 40 minutes; about 45 minutes; about 50 minutes; about 55 minutes; or about 60 minutes. In some embodiments the incubation time is proportional to tumor fragment density. In some embodiments the incubation time is proportional to tumor fragment surface to volume ratio.

[00357] In some embodiments, a tumor fragment with a cross section of about 1.5 mm × 1.5 mm is incubated in cryopreservation medium for about 20 minutes, for about 30 minutes, for about 35 minutes, for about 40 minutes, or for less than about 60 minutes. In some

embodiments, a tumor fragment with a cross section of about $2\text{ mm} \times 2\text{ mm}$ is incubated in cryopreservation medium for about 20 minutes, for about 30 minutes, for about 35 minutes, for about 40 minutes, for about 45 minutes, for about 50 minutes, or for about 60 minutes. In some embodiments, a tumor fragment with a cross section of about $3\text{ mm} \times 3\text{ mm}$ is incubated in cryopreservation medium for at least 20 minutes, for about 30 minutes, for about 35 minutes, for about 40 minutes, for about 45 minutes, for about 50 minutes, or for about 60 minutes. In some embodiments, a tumor fragment with a cross section of about $4\text{ mm} \times 4\text{ mm}$ is incubated in cryopreservation medium for about 30 minutes, for about 35 minutes, for about 40 minutes, for about 45 minutes, for about 50 minutes, for about 60 minutes, or for about 70 minutes. In some embodiments, a tumor fragment with a cross section of about $5\text{ mm} \times 5\text{ mm}$ is incubated in cryopreservation medium for about 30 minutes, for about 35 minutes, for about 40 minutes, for about 45 minutes, for about 50 minutes, for about 60 minutes, or for about 70 minutes. In some embodiments, a tumor fragment with a cross section of about $6\text{ mm} \times 6\text{ mm}$ is incubated in cryopreservation medium for at least 20 minutes, for about 30 minutes, for about 40 minutes, for about 45 minutes, for about 50 minutes, for about 60 minutes, or for about 70 minutes.

[00358] In some embodiments, the storage medium comprises about 5% v/v DMSO and the tumor tissue is incubated for about 60 minutes in a storage medium at a temperature in the range from about 2°C to about 8°C before freezing. In some embodiments, the storage medium comprises about 10% v/v DMSO and the tumor tissue is incubated for at least about 30 minutes to about 60 minutes in a storage medium at a temperature in the range from about 2°C to about 8°C before freezing.

[00359] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises incubating cryopreserved tumor fragments in a culture medium comprising IL-2, wherein the number of TILs are expanded. In some embodiments, the number of TILs is expanded 100-fold. In some embodiments, the number of TILs is expanded 150-fold. In some embodiments, the number of TILs is expanded 200-fold. In some embodiments, the number of TILs is expanded 250-fold. In some embodiments, the number of TILs is expanded about 300-fold. In some embodiments, the number of TILs is expanded about 350-fold. In some embodiments, the number of TILs is expanded about 400-fold. In some embodiments, the number of TILs is expanded about 450-fold. In some embodiments, the number of TILs is expanded about 500-fold. In some embodiments, the number of TILs is expanded about 550-

fold. In some embodiments, the number of TILs is expanded about 600-fold. In some embodiments, the number of TILs is expanded about 700-fold. In some embodiments, the number of TILs is expanded about 750-fold. In some embodiments, the number of TILs is expanded about 800-fold. In some embodiments, the number of TILs is expanded about 850-fold. In some embodiments, the number of TILs is expanded about 900-fold. In some embodiments, the number of TILs is expanded about 1000-fold. In some embodiments, the number of TILs is expanded about 1200-fold. In some embodiments, the number of TILs is expanded about 1500-fold. In some embodiments, the number of TILs is expanded about 1600-fold. In some embodiments, the number of TILs is expanded about 2000-fold. In some embodiments, the number of TILs is expanded about 2100-fold. In some embodiments, the number of TILs is expanded about 2200-fold. In some embodiments, the number of TILs is expanded about 2500-fold. In some embodiments, the number of TILs is expanded greater than 2500-fold.

[00360] In some embodiments, a method for manufacturing TILs from frozen tumor tissue comprises:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
 - (iv) freezing the vessel; and
 - (v) storing the vessel such that the vessel remains frozen;
- (b) thawing the vessel;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (d) removing at least a plurality of the TILs; and

- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00361] In some embodiments, a method for manufacturing TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
- (e) freezing the vessel;
- (f) storing the vessel such that the vessel remains frozen;
- (g) thawing the vessel;
- (h) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (i) removing at least a plurality of the TILs; and
- (j) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00362] In some embodiments, the method of manufacturing TILs from a cryopreserved tumor fragment, further comprises addition to OKT-3 to the first culture medium.

[00363] In some embodiments, the fresh tumor tissue is trimmed to between 1.5 mm and 6 mm in diameter and between 1.5 mm and 6 mm in thickness. In yet other embodiments, the fresh tumor tissue is trimmed to about 6 mm × 6 mm × 6 mm. In some embodiments, the fresh tumor tissue is trimmed into fragments with a cross section of about 1.5 mm × 1.5 mm, about 2 mm × 2 mm, about 2.5 mm × 2.5 mm, about 3 mm × 3 mm, about 3.5 mm × 3.5 mm, about 4 mm × 4

mm, about 4.5 mm × 4.5 mm, about 5 mm × 5 mm, about 5.5 mm × about 5.5 mm, or about 6 mm × 6 mm.

[00364] In some embodiments, the storage medium further comprises an antibacterial agent. In some embodiments the antibacterial agent is gentamicin. In some embodiments the storage medium comprises gentamicin at a concentration of at least 20 µg/mL. In some embodiments the storage medium comprises gentamicin at a concentration of at least 50 µg/mL. In some embodiments the storage medium comprises an antibacterial agent, an antifungal agent, and combinations thereof. In some embodiments, the antifungal agent is amphotericin B and is present from about 0.25 µg/mL to about 2.5 µg/mL. In some embodiments, the antifungal agent is Fungin™ and is present from about 5 µg/mL to about 50 µg/mL.

[00365] In some embodiments, the tumor tissue is washed in a physiologically buffered isotonic saline solution. In some embodiments, the washing comprises three serial washes of at least three minutes each, with the physiologically buffered isotonic saline solution replaced after each serial wash. In some embodiments, the physiologically buffered isotonic saline solution comprises Hank's Balance Salt Solution (HBSS). In some embodiments, the physiologically buffered isotonic saline solution comprises tris-buffered saline (TBS). In some embodiments, the physiologically buffered isotonic saline solution comprises phosphate buffered saline (PBS). In some embodiments, the physiologically buffered isotonic saline solution comprises Dulbecco's phosphate-buffered saline (DPBS). In some embodiments, the physiologically buffered isotonic saline solution in one serial wash may be a different physiologically buffered isotonic saline solution than used in one or more of the other serial washes.

[00366] In some embodiments the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[00367] In some embodiments, the freezing step comprises freezing the vessel at about -125°C to about -195°C. In some embodiments, the vessel is frozen at a temperature from about -125°C to about -150°C; in some embodiments the vessel is frozen at a temperature from about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at a temperature from about -135°C.

[00368] In some embodiments, the method is performed with a fresh tumor sample from a human subject.

[00369] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC) tissue, and HPV-positive tumor tissue.

[00370] In some embodiments, the method further comprises a split between the first expansion (or first culture) step and the second expansion (or second culture) step. In an embodiment, the split occurs on about day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some embodiments, steps (c) through (e) are complete in about 22 days. In yet other embodiments, steps (c) through (e) or steps (h) through (j), as applicable, are complete in about 21 days; in some embodiments steps (c) through (e) or steps (h) through (j), as applicable, are complete in about 20 days; and in some embodiments steps (c) through (e) or steps (h) through (j), as applicable, are complete in about 16 days.

[00371] In some embodiments, the first culture medium comprises OKT-3.

[00372] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof, such methods comprising:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;

- (b) thawing the tumor tissue;
- (c) adding the tumor tissue into a closed system;
- (d) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (c) to step (d) occurs without opening the system;
- (e) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (d) to step (e) occurs without opening the system;
- (f) harvesting the therapeutic population of TILs obtained from step (f), wherein the transition from step (e) to step (f) occurs without opening the system;
- (g) transferring the harvested TIL population from step (f) to an infusion bag, wherein the transfer from step (f) to (g) occurs without opening the system;
- (h) cryopreserving the infusion bag comprising the harvested TIL population from step (g) using a cryopreservation process; and
- (i) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

[00373] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof are provided, such methods comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

- (a) obtaining tumor tissue from the subject;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;
- (e) freezing the vessel using the vapor phase of liquid nitrogen;
- (f) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (g) thawing the tumor tissue;
- (h) adding the tumor tissue into a closed system;
- (i) performing in the closed system a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (h) to step (i) occurs without opening the system;
- (j) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (i) to step (j) occurs without opening the system;
- (k) harvesting the therapeutic population of TILs obtained from step (j), wherein the transition from step (j) to step (k) occurs without opening the system;

- (l) transferring the therapeutic of TILs harvested in step (k) to an infusion bag, wherein the transfer from step (k) to (l) occurs without opening the system;
- (m) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (l) using a cryopreservation process;
- (n) thawing the therapeutic population of TILs in the infusion bag from step (m); and
- (o) administering a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (n) to the subject.

[00374] 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, endometrial cancer, thyroid cancer, colorectal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, melanoma, refractory melanoma, metastatic melanoma, 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.

[00375] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising incubating the tumor tissue for 30 minutes in a storage medium at 2°C - 8°C before freezing;
- (c) thawing the tumor tissue;
- (d) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (e) removing at least a plurality of the TILs; and
- (f) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00376] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) incubating the tumor tissue for 30 minutes in a storage medium at 2°C - 8°C;
- (c) storing the tumor tissue in a frozen state;
- (d) thawing the tumor tissue;
- (e) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (f) removing at least a plurality of the TILs; and
- (g) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00377] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;
 - (iv) freezing the vessel; and
 - (v) storing the vessel such that the vessel remains frozen;
- (c) thawing the vessel;

- (d) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (e) removing at least a plurality of the TILs; and
- (f) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00378] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;
- (e) freezing the vessel;
- (f) storing the vessel such that the vessel remains frozen;
- (g) thawing the vessel;
- (h) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (i) removing at least a plurality of the TILs; and
- (j) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00379] In some embodiments, the tumor tissue is washed in a physiologically buffered isotonic saline solution. In some embodiments, the washing comprises three serial washes of at least three minutes each, with the physiologically buffered isotonic saline solution replaced after each serial wash. In some embodiments, the physiologically buffered isotonic saline solution comprises Hank's Balance Salt Solution (HBSS). In some embodiments, the physiologically

buffered isotonic saline solution comprises tris-buffered saline (TBS). In some embodiments, the physiologically buffered isotonic saline solution comprises phosphate buffered saline (PBS). In some embodiments, the physiologically buffered isotonic saline solution comprises Dulbecco's phosphate-buffered saline (DPBS). In some embodiments, the physiologically buffered isotonic saline solution in one serial wash may be a different physiologically buffered isotonic saline solution than used in one or more of the other serial washes.

[00380] In some embodiments the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[00381] In some embodiments, the freezing step comprises freezing the vessel at about -125°C to about -195°C. In some embodiments, the vessel is frozen at a temperature from about -125°C to about -150°C; in some embodiments the vessel is frozen at a temperature from about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at a temperature of about -135°C.

[00382] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC) tissue, and HPV-positive tumor tissue.

[00383] In some embodiments, the method further comprises a split between the first expansion (or first culture) step and the second expansion (or second culture) step. In an embodiment, the split occurs on about day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some embodiments, steps (d) through (f) or steps (h) through (j), as applicable, are complete in about 22 days. In yet other embodiments, steps (d) through (f) or steps (h) through (j), as applicable, are complete in about 21 days; in some embodiments steps (d) through (f) or steps (h) through (j), as applicable, are complete in about 20 days; and in some embodiments steps (d) through (f) or steps (h) through (j), as applicable, are complete in about 16 days.

[00384] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof, such methods comprising:

- (a) obtaining tumor tissue from the subject, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 to about 70 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (c) thawing the tumor tissue;
- (d) adding the tumor tissue into a closed system;
- (e) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (d) to step (e) occurs without opening the system;
- (f) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed

container providing a second gas-permeable surface area, and wherein the transition from step (e) to step (f) occurs without opening the system;

- (g) harvesting the therapeutic population of TILs obtained from step (f), wherein the transition from step (f) to step (g) occurs without opening the system;
- (h) transferring the harvested TIL population from step (g) to an infusion bag, wherein the transfer from step (g) to (h) occurs without opening the system;
- (i) cryopreserving the infusion bag comprising the harvested TIL population from step (h) using a cryopreservation process; and
- (j) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (i) to the subject.

[00385] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof, such methods comprising:

- (a) obtaining tumor tissue from the subject, wherein the tumor tissue comprises TILs;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 to about 70 minutes;
- (e) freezing the vessel using the vapor phase of liquid nitrogen;
- (f) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (g) thawing the tumor tissue;
- (h) adding the tumor tissue into a closed system;
- (i) performing in the closed system a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first

- population of TILs, and wherein the transition from step (h) to step (i) occurs without opening the system;
- (j) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (i) to step (j) occurs without opening the system;
 - (k) harvesting the therapeutic population of TILs obtained from step (j), wherein the transition from step (j) to step (k) occurs without opening the system;
 - (l) transferring the therapeutic population of TILs harvested in step (k) to an infusion bag, wherein the transfer from step (k) to (l) occurs without opening the system;
 - (m) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (l) using a cryopreservation process;
 - (o) thawing the therapeutic population of TILs in the infusion bag from step (m); and
 - (p) administering a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (o) to the subject.

[00386] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;

- (iv) freezing the vessel; and
- (v) storing the vessel such that the vessel remains frozen;
- (b) thawing the vessel;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (d) removing at least a plurality of the TILs; and
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00387] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;
- (e) freezing the vessel;
- (f) storing the vessel such that the vessel remains frozen;
- (g) thawing the vessel;
- (h) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (i) removing at least a plurality of the TILs; and
- (j) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00388] In some embodiments, the fresh tumor tissue is trimmed to between 1.5 mm and 6 mm

in diameter and between about 1.5 mm and about 6 mm in thickness. In yet other embodiments, the fresh tumor tissue is trimmed to about 6 mm × 6 mm × 6 mm.

[00389] In some embodiments, the storage medium further comprises an antibacterial agent. In some embodiments the antibacterial agent is gentamicin. In some embodiments the storage medium comprises gentamicin at a concentration of at least 20 µg/mL. In some embodiments the storage medium comprises gentamicin at a concentration of at least 50 µg/mL. In some embodiments the storage medium comprises an antibacterial agent, an antifungal agent, and combinations thereof. In some embodiments, the antifungal agent is amphotericin B and is present from about 0.25 µg/mL to about 2.5 µg/mL. In some embodiments, the antifungal agent is fungin and is present from about 5 µg/mL to about 50 µg/mL.

[00390] In some embodiments, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing the trimming step of the method. In some embodiments, the washing comprises at least three serial washes of at least three minutes each, wherein the HBSS is replaced after each wash.

[00391] In some embodiments, thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[00392] In some embodiments, the freezing step comprises freezing the vessel at about -125°C to about -195°C. In some embodiments, the vessel is frozen at about -125°C to about -150°C; in some embodiments the vessel is frozen at about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at about -135°C.

[00393] In some embodiments, the method is performed with a fresh tumor sample from a human subject.

[00394] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC), and HPV-positive tumor tissue.

[00395] In some embodiments, the method further comprises a split between the first expansion

(or first culture) step and the second expansion (or second culture) step. In an embodiment, the split occurs on day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some embodiments, steps (c) through (e) or steps (h) through (j), as applicable, are complete in about 22 days. In yet other embodiments, steps (c) through (e) or steps (h) through (j), as applicable, are complete in 21 days; in some embodiments steps (c) through (e) or steps (h) through (j), as applicable, are complete in about 20 days; and in some embodiments steps (c) through (e) or steps (h) through (j), as applicable, are complete in about 16 days.

[00396] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof, such methods comprising:

- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 20 to about 70 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (b) thawing the tumor tissue;
- (c) adding the tumor fragments into a closed system;
- (d) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold

greater in number than the first population of TILs, and wherein the transition from step (c) to step (d) occurs without opening the system;

- (e) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (d) to step (e) occurs without opening the system;
- (f) harvesting the therapeutic population of TILs obtained from step (f), wherein the transition from step (e) to step (f) occurs without opening the system;
- (g) transferring the harvested TIL population from step (f) to an infusion bag, wherein the transfer from step (f) to (g) occurs without opening the system;
- (h) cryopreserving the infusion bag comprising the harvested TIL population from step (g) using a cryopreservation process; and
- (i) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

[00397] In some embodiments, the invention provides methods of treating cancer for a human subject in need thereof, such methods comprising:

- (a) obtaining tumor tissue from the subject;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 20 to about 70 minutes;
- (e) freezing the vessel using the vapor phase of liquid nitrogen;
- (f) storing the vessel at the vapor phase of liquid nitrogen temperature;

- (g) thawing the tumor tissue;
- (h) adding the tumor tissue into a closed system;
- (i) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (h) to step (i) occurs without opening the system;
- (j) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (i) to step (j) occurs without opening the system;
- (k) harvesting the therapeutic population of TILs obtained from step (j), wherein the transition from step (j) to step (k) occurs without opening the system;
- (l) transferring the therapeutic population of TILs harvested in step (k) to an infusion bag, wherein the transfer from step (k) to (l) occurs without opening the system;
- (m) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (l) using a cryopreservation process;
- (n) thawing the therapeutic population of TILs in infusion bag from step (m); and
- (o) administering a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (n) to the subject.

[00398] 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,

endometrial cancer, thyroid cancer, colorectal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, melanoma, refractory melanoma, metastatic melanoma, 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.

[00399] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising incubating the tumor tissue for about 20 to about 70 minutes in a storage medium at a temperature in the range from about 2°C to about 8°C before freezing;
- (c) thawing the tumor tissue;
- (d) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (e) removing at least a plurality of the TILs; and
- (f) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00400] The invention provides a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) incubating the tumor tissue for about 20 to about 70 minutes in a storage medium at a temperature in the range from about 2°C to about 8°C;
- (c) storing the tumor tissue in a frozen state;
- (d) thawing the tumor tissue;
- (e) treating the tumor tissue in a gas permeable container with a first cell culture medium

and interleukin 2 (IL-2) to provide TILs;

- (f) removing at least a plurality of the TILs; and
- (g) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00401] In some embodiments, a method for expanding TILs from frozen tumor tissue comprises:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 30 minutes;
 - (iv) freezing the vessel; and
 - (v) storing the vessel such that the vessel remains frozen;
- (c) thawing the vessel;
- (d) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (e) removing at least a plurality of the TILs; and
- (f) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

[00402] In some embodiments, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing the tissue trimming step. In some embodiments, the washing comprises at least three serial washes of at least three minutes each, wherein the HBSS

is replaced after each wash.

[00403] In some embodiments the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[00404] In some embodiments, the freezing step comprises freezing the vessel at a temperature between about -125°C to about -195°C. In some embodiments, the vessel is frozen at a temperature between about -125°C to about -150°C; in some embodiments the vessel is frozen at a temperature between about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at a temperature of about -135°C.

[00405] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC), and HPV-positive tumor tissue.

[00406] In some embodiments, the method further comprises a split between the first expansion (or first culture) step and the second expansion (or second culture) step. In an embodiment, the split occurs on day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some embodiments, steps (d) through (f) or steps (e) through (g), as applicable, are complete in about 22 days. In yet other embodiments, steps (d) through (f) or steps (e) through (g), as applicable, are complete in about 21 days; in some embodiments steps (d) through (f) or steps (e) through (g), as applicable, are complete in about 20 days; and in some embodiments steps (d) through (f) or steps (e) through (g), as applicable, are complete in about 16 days.

[00407] In some embodiments, the invention provides for methods of treating cancer for a human subject in need thereof, such methods comprising:

- (a) obtaining tumor tissue from the subject, wherein the tumor tissue comprises TILs;
- (b) storing the tumor tissue in a frozen state, the method of storing the tumor tissue

comprising:

- (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range from about 2°C to about 8°C for about 20 minutes to about 70 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (c) thawing the tumor tissue;
- (d) adding the tumor tissue into a closed system;
- (e) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (d) to step (e) occurs without opening the system;
- (f) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (e) to step (f) occurs without opening the system;
- (g) harvesting the therapeutic population of TILs obtained from step (f), wherein the transition from step (f) to step (g) occurs without opening the system;

- (h) transferring the harvested TIL population from step (g) to an infusion bag, wherein the transfer from step (g) to (h) occurs without opening the system;
- (i) cryopreserving the infusion bag comprising the harvested TIL population from step (h) using a cryopreservation process; and
- (j) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (i) to the subject.

[00408] In some embodiments, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing step (i) of the tumor tissue storing method. In some embodiments, the washing comprises at least three serial washes of at least three minutes each, wherein the HBSS is replaced after each wash.

[00409] In some embodiments step (c) comprises immersing the vessel in a 37°C water bath for about 5 minutes.

[00410] In some embodiments, step (iv) of the tumor tissue storing method comprises freezing the vessel at a temperature between about -125°C to about -195°C. In some embodiments, the vessel is frozen at a temperature between about -125°C to about -150°C; in some embodiments the vessel is frozen at a temperature between about -125°C to about -145°C; in yet further embodiments, the vessel is frozen at a temperature of about -135°C.

[00411] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, endometrial tumor tissue, thyroid tumor tissue, ovarian tumor tissue, head and neck squamous cell carcinoma (HNSCC), and HPV-positive tumor tissue.

[00412] In some embodiments, the method further comprises a split between the first expansion (or first culture) step and the second expansion (or second culture) step. In an embodiment, the split occurs on day 16. In some embodiments, the first culture step is complete in about 11 days. In other embodiments, the first expansion (or first culture) step is complete in about 7 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 7 days and the second expansion (or second culture) step is complete in about 14 days. In some

embodiments, steps (e) through (g) are complete in about 22 days. In yet other embodiments, steps (e) through (g) are complete in about 21 days; in some embodiments steps (e) through (g) are complete in about 20 days; and in some embodiments steps (e) through (g) are complete in about 16 days.

[00413] In some embodiments, the first expansion (or first culture) step is complete in about 5 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 3 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 5 days and the second expansion (or second culture) step is complete in about 11 days. In yet other embodiments, the first expansion (or first culture) step is complete in about 3 days and the second expansion (or second culture) step is complete in about 13 days. Optionally, the second expansion (or second culture) can be split into two or more cultures on or about the fifth day or sixth day of the second expansion (or second culture).

[00414] In some embodiments of the above methods to manufacture or expand TILs, OKT-3 is present in the culture medium beginning at day 0.

[00415] In some embodiments, the invention provides any of the foregoing methods of treating cancer in a subject modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days.

[00416] In some embodiments, the invention provides any of the foregoing methods of treating cancer in a subject modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days.

[00417] In some embodiments, the invention provides any of the foregoing methods of treating cancer in a subject modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days and the second expansion (or second culture) is complete in about 11 days.

[00418] In some embodiments, the invention provides any of the foregoing methods of treating cancer in a subject modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days, the second expansion (or second culture) is complete in about 11 days, and the second expansion (or second culture) is split into two or more cultures on or about the fifth day of the second expansion (or second

culture).

[00419] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days and the second expansion (or second culture) is complete in about 13 days.

[00420] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days, the second expansion (or second culture) is complete in about 13 days, and the second expansion (or second culture) is split into two or more cultures on or about the sixth day of the second expansion (or second culture).

[00421] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days.

[00422] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days.

[00423] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days and the second expansion (or second culture) is complete in about 11 days.

[00424] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days, the second expansion (or second culture) is complete in

about 11 days, and the second expansion (or second culture) is split into two or more cultures on or about the fifth day of the second expansion (or second culture).

[00425] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days and the second expansion (or second culture) is complete in about 13 days.

[00426] In some embodiments, the invention provides any of the foregoing methods of expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days, the second expansion (or second culture) is complete in about 13 days, and the second expansion (or second culture) is split into two or more cultures on or about the sixth day of the second expansion (or second culture).

[00427] In some embodiments, the invention provides any of the foregoing methods for preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days and the second expansion (or second culture) is complete in about 13 days.

[00428] In some embodiments, the invention provides any of the foregoing methods of preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days, the second expansion (or second culture) is complete in about 13 days, and the second expansion (or second culture) is split into two or more cultures on or about the sixth day of the second expansion (or second culture).

[00429] In some embodiments, the invention provides any of the foregoing methods of preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days.

[00430] In some embodiments, the invention provides any of the foregoing methods of

preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days.

[00431] In some embodiments, the invention provides any of the foregoing methods of preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days and the second expansion (or second culture) is complete in about 11 days.

[00432] In some embodiments, the invention provides any of the foregoing methods of preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 5 days, the second expansion (or second culture) is complete in about 11 days, and the second expansion (or second culture) is split into two or more cultures on or about the fifth day of the second expansion (or second culture).

[00433] In some embodiments, the invention provides any of the foregoing methods of preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days and the second expansion (or second culture) is complete in about 13 days.

[00434] In some embodiments, the invention provides any of the foregoing methods of preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy modified as appropriate such that in the step of the first expansion (or first culture) the first expansion (or first culture) is complete in about 3 days, the second expansion (or second culture) is complete in about 13 days, and the second expansion (or second culture) is split into two or more cultures on or about the sixth day of the second expansion (or second culture).

[00435] In some embodiments, the invention provides any of the foregoing methods modified as appropriate such that the first expansion (or first culture) is performed in a defined medium.

[00436] In some embodiments, the invention provides any of the foregoing methods modified as appropriate such that the second expansion (or second culture) is performed in a defined medium.

[00437] In some embodiments, the invention provides any of the foregoing methods modified as appropriate such that the first expansion (or first culture) and the second expansion (or second culture) are performed in defined media that are the same or different.

TIL Manufacturing Processes

[00438] There are various methods to expand TILs known to one skilled in the art. For example, Jin *et al.*, *J. Immunother.* **35**(3): 283-292 (2012), “Simplified Method of the Growth of Human Tumor Infiltrating Lymphocytes in Gas-permeable Flasks to Numbers Needed for Patient Treatment,” the disclosure of which is incorporated by reference herein, teaches simplified methods of producing TILs for clinical use. Jin *et al.* teaches a first TIL culture followed by a rapid expansion (REP) protocol, which combined, enables one skilled in the art to produce clinically useful quantities of TILs. In some embodiments, the invention provides a method of manufacturing TILs comprising the step of cryopreserving a tumor, thawing a tumor, and performing the process described in Jin *et al.* Briefly, this process involves the following process. TILs may initially be cultured from enzymatic tumor digests and tumor fragments (about 1 to 8 mm³) produced by sharp dissection. Tumor digests are produced by incubation in enzyme media (RPMI 1640, 2 mM GlutaMAX, 10 mg/mL gentamicin, 30U/mL DNase, and 1.0 mg/mL collagenase) followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). Immediately after placing the tumor in enzyme media, it is mechanically dissociated for approximately 1 minute. The material was then incubated for 30 minutes at 37°C in 5% CO₂ and is then mechanically disrupted again for approximately 1 minute and incubated again for 30 minutes at 37°C in 5% CO₂. The tumor is then mechanically disrupted a third time for approximately 1 minute. If after the third mechanical disruption, large pieces of tissue were present, 1 or 2 additional mechanical dissociations may be applied to the sample, with or without 30 additional minutes of incubation at 37°C in 5% CO₂. 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 may be performed to remove these cells. When TIL cultures are initiated in 24-well plates (Costar 24-well cell culture cluster, flat bottom; Corning Incorporated, Corning, NY), each well is seeded with 1×10⁶ tumor digest cells or one tumor fragment approximately about 1 to 8 mm³ in size in 2 mL of complete medium (CM) with IL-2 (6000 IU/mL; Chiron Corp., Emeryville, CA). CM comprised RPMI 1640 with GlutaMAX, supplemented with 10%

human AB serum, 25mM Hepes, and about 10 µg/mL gentamicin. When cultures were initiated in gas-permeable flasks with a 40 mL capacity and a 10 cm² gas-permeable silicon bottom (G-Rex10; Wilson Wolf Manufacturing, New Brighton, MN), each flask is loaded with 10 to 40×10⁶ viable tumor digest cells or 5 to 30 tumor fragments in 10 to 40 mL of CM with IL-2. Both the G-Rex10 and 24-well plates are incubated in a humidified incubator at 37°C in 5%CO₂ and 5 days after culture initiation, half the media is removed and replaced with fresh CM and IL-2 and after day 5, half the media is changed every 2–3 days. REP of TIL is performed using T-175 flasks and gas-permeable bags or gas-permeable G-Rex flasks. For TIL REP in T-175 flasks, 1×10⁶ TILs suspended in 150 mL of media was added to each T-175 flask. The TIL were cultured with irradiated (50 Gy) allogeneic PBMC as “feeder” cells at a ratio of 1 to 100 and the cells are 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% CO₂. Half the media is changed on day 5 using 50/50 medium with 3000 IU/mL of IL-2. On day 7, cells from 2 T-175 flasks are combined in a 3L 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 was counted every day or 2 and fresh media was added to keep the cell count between 0.5 and 2.0×10⁶ cells/mL. For TIL REP in 500mL capacity flasks with 100 cm² gas-permeable silicon bottoms (G-Rex100, Wilson Wolf), 5×10⁶ to 10×10⁶ 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. G-Rex100 flasks are incubated at 37°C in 5% CO₂. On day 5, 250 mL of supernatant is removed and placed into centrifuge bottles and centrifuged at 1500 rpm (491 g) for 10 minutes. TIL pellets are resuspended with 150 mL of fresh 50/50 medium with 3000 IU/mL of IL-2 and added back to the original G-Rex100 flasks. When TIL are expanded serially in G-Rex100 flasks, on day 7 the TIL in each G-Rex100 were suspended in the 300 mL of media present in each flask and the cell suspension was divided into three 100 mL aliquots that were used to seed 3 G-Rex100 flasks. 150 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to each flask. The G-Rex100 flasks are incubated at 37°C in 5% CO₂ and after 4 days 150 mL of AIM-V with 3000 IU/mL of IL-2 was added to each G-Rex100 flask. The cells are harvested on day 14 of culture.

[00439] In some embodiments, the expansion methods according to the present invention comprises preparing expansion of cells from frozen tumor. Tumor samples are collected from patients and cryopreserved according to methods disclosed herein. When ready to expand the cells, for a process performed at the 1/100th scale, about six 2-3 mm diameter frozen tumor fragments are thawed using a 37°C water bath for 5 + 1 minutes and washed in sterile Hanks Balanced Salt Solution (HBSS) supplemented with gentamicin. The fragments are then placed into a G-Rex 10M flask (Wilson Wolf Mfg., New Brighton, MN) or other gas permeable container with CM1 media or a serum-free or defined media containing about 6,000 IU/mL of rhIL-2. These pre-REP (or first expansion) cultures are incubated for 5 days in a 37°C incubator. On day 5, the pre-REP cells are harvested, and the REP (or second expansion) is initiated by co-culturing 10% of the pre-REP TILs with either 25×10^6 or 50×10^6 PBMC feeder cells in CM2 media or a serum-free or defined media containing 3,000 IU/mL rhIL-2 and 30 ng/mL of OKT-3 in a G-Rex 5M flask (Wilson Wolf Mfg., New Brighton, MN) or other gas permeable container. On day 10, the culture is split into G-Rex 5M flasks or other gas permeable container containing no more than 10×10^6 cells. The split cultures are incubated for 6 additional days in CM4 media or other serum-free or defined media containing 3,000 IU/mL rhIL-2. On day 16, the cells are harvested and frozen using CryoStor10 (Biolife, USA). This process is referred to herein as the “Early REP Method 1” process or “ER-1.”

[00440] In some embodiments, the expansion methods according to the present invention comprises preparing expansion of cells from frozen tumor. Tumor samples are collected from patients and cryopreserved according to methods disclosed herein. When ready to expand the cells, for a process performed at the 1/100th scale, about six 2-3 mm diameter frozen tumor fragments are thawed using a 37°C water bath for 5 + 1 minutes and washed in sterile Hanks Balanced Salt Solution (HBSS) supplemented with gentamicin. The fragments are then placed into a G-Rex 10M flask (Wilson Wolf Mfg., New Brighton, MN) or other gas permeable container with CM1 media or a serum-free or defined media containing about 6,000 IU/mL of rhIL-2. These pre-REP (or first expansion) cultures are incubated for 3 days in a 37°C incubator. On day 3, the pre-REP cells are harvested, and the REP (or second expansion) is initiated by co-culturing 10% of the pre-REP TILs with either 25×10^6 or 50×10^6 PBMC feeder cells in CM2 media or a serum-free or defined media containing 3,000 IU/mL rhIL-2 and 30 ng/mL of OKT-3

in a G-Rex 5M flask (Wilson Wolf Mfg., New Brighton, MN) or other gas permeable container. On day 9, the culture is split into G-Rex 5M flasks or other gas permeable container containing no more than 10×10^6 cells. The split cultures are incubated for 7 additional days in CM4 media or other serum-free or defined media containing 3,000 IU/mL rhIL-2. On day 16, the cells are harvested and frozen using CryoStor10 (Biolife, USA). This process is referred to herein as the “Early REP Method 2” process or “ER-2.”

[00441] In some embodiments, the invention provides a method of manufacturing TILs comprising the step of cryopreserving a tumor, thawing a tumor, and performing the following process. TILs can be produced by rapid expansion using stimulation of peripheral blood mononuclear cells (PBMC) *in vitro* with an antigen (one or more, including antigenic portions thereof, such as epitope(s), or a cell) of the cancer, which can be optionally expressed from a vector, such as an HLA-A2 binding peptide, e.g., 0.3 μ M MART-1:26-35 (27L) or gp100:209-217 (210M), in the presence of a T-cell growth factor, such as 300 IU/mL IL-2 or IL-15, with IL-2 being preferred. The *in vitro*-induced TILs are 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 re-stimulated with irradiated, autologous lymphocytes or with irradiated HLA-A2⁺ allogeneic lymphocytes and IL-2, for example. TILs can be selected for highly avid recognition of any of the unique antigens produced as a result of the estimated 10,000 genetic mutations encoded by each tumor cell genome. The antigen, however, need not be unique. T-cells can be selected for highly avid recognition of one or more antigens of a cancer, including an antigenic portion of one or more antigens, such as an epitope, or a cell of the cancer. An “antigen of a cancer” and an “antigen of the cancer” are intended to encompass all of the aforementioned antigens. If the cancer is melanoma, such as metastatic melanoma, preferably the TILs are selected for highly avid recognition of MART-1 (such as MART-1 :26-35 (27L)), gp100 (such as gp100:209-217 (210M)), or a “unique” or patient-specific antigen derived from a tumor encoded mutation. Other suitable melanoma antigens for which highly avid recognition by TILs can be selected include, but are not limited to, tyrosinase, tyrosinase related protein (TRP)1, TRP2, and MAGE. Antigens, such as NY-ESO-1, telomerase, p53, HER2/neu, carcinoembryonic antigen, or prostate-specific antigen, can be used to select for highly avid recognition by TILs for treatment of lung carcinoma, breast cancer, colon cancer, prostate cancer, and the like TILs can be selected include, but are not limited to, tyrosinase, tyrosinase

related protein.

[00442] 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), 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. The cryopreserved tumor fragments are suitable starting points for initial or first cultures to manufacture TILs for therapeutic or other purposes.

[00443] An exemplary TIL process known as process 2A containing some of these features is depicted in Figure 1. Another exemplary TIL process, known as process 1C, is described and compared to process 2A in Figures 5 and 6. An embodiment of process 2A is shown Figure 1.

[00444] 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.

[00445] In some embodiments, the TILs may be cryopreserved and thawed for administration to a patient. Once thawed, they may also be restimulated to increase their metabolism prior to infusion into a patient.

[00446] In some embodiments, the first expansion (including processes referred to as the preREP as well as processes shown in Figure 1 as Step A) is shortened to 3 to 14 days and the second expansion (including processes referred to as the REP as well as processes shown in Figure 1 as Step B) is shortened to 7 to 14 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the first expansion (for example, an expansion described as Step B in Figure 1) is shortened to 11 days and the second expansion (for example, an expansion as described in Step D in Figure 1) is shortened to 11 days. In some embodiments,

the combination of the first expansion and second expansion (for example, expansions described as Step B and Step D in Figure 1) is shortened to 22 days, as discussed in detail below and in the examples and figures.

[00447] The “Step” Designations A, B, C, *etc.*, below are in reference to Figure 1 and in reference to certain embodiments described herein. The ordering of the Steps below and in Figure 1 is exemplary and any combination or order of steps, as well as additional steps, repetition of steps, and/or omission of steps is contemplated by the present application and the methods disclosed herein.

Step A. Obtain Patient Tumor Sample

[00448] 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.

[00449] 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.

[00450] 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.

[00451] 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.

[00452] Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 mm³, with from about 2-3 mm³ 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% CO₂, 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.

[00453] In general, the harvested cell suspension is called a “primary cell population” or a “freshly harvested” cell population.

[00454] 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.

[00455] In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained in, for example, Step A (as provided in Figure 1). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3 . In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 mm^3 to about 1500 mm^3 . In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3 . In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams. In some embodiments, the multiple fragments comprise about 4 fragments.

[00456] 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 mm³. In some embodiments, the tumors are 1-4 mm × 1-4 mm × 1-4 mm. In some embodiments, the tumors are 1 mm × 1 mm × 1 mm. In some embodiments, the tumors are 2 mm × 2 mm × 2 mm. In some embodiments, the tumors are 3 mm × 3 mm × 3 mm. In some embodiments, the tumors are 4 mm × 4 mm × 4 mm. In embodiments wherein the tumor tissue is first frozen before initiating a culture, tumors are about 6 mm × 6 mm × 6 mm.

[00457] In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of fatty tissue on each piece.

[00458] In some embodiments, the tumor fragmentation is performed in order to maintain the tumor internal structure. In some embodiments, the tumor fragmentation is performed without performing a sawing motion with a scalpel. In some embodiments, the TILs are obtained from tumor digests. In some embodiments, tumor digests were generated by incubation in enzyme media, for example but not limited to RPMI 1640, 2 mM GlutaMAX, 10 µg/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% CO₂ and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% CO₂, 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% CO₂. 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.

[00459] 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.

[00460] In some embodiments, cells can be optionally frozen after sample harvest and stored frozen prior to entry into the expansion described in Step B, which is described in further detail below, as well as exemplified in Figure 1.

Step B: First Expansion

[00461] 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 (for example “older TILs” have further undergone more rounds of ex vivo replication prior to administration to a subject/patient). Features of young TILs have been described in the literature, for example Donia, *et 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.

[00462] The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using methods referred to as process 1C, as exemplified in Figure 5 and/or Figure 6. In some embodiments, the TILs obtained in the first expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin 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.*, TCR α/β).

[00463] After dissection or digestion of tumor fragments, for example such as described in Step A of Figure 1, the resulting cells are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 3 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 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.

[00464] In a preferred embodiment, expansion of TILs may be performed using an initial bulk TIL expansion step (for example such as those described in Step B of Figure 1, which can include processes referred to as pre-REP) as described below and herein, followed by a second expansion (Step D, including processes referred to as rapid expansion protocol (REP) steps) as described below under Step D and herein, followed by optional cryopreservation, and followed by a second Step D (including processes referred to as restimulation REP steps) as described below and herein. The TILs obtained from this process may be optionally characterized for phenotypic characteristics and metabolic parameters as described herein.

[00465] 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 .

[00466] 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 $\mu\text{g/mL}$ 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 10×10^6 to 40×10^6 viable tumor digest cells or 5 to 30 tumor fragments in 10 to 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 to 3 days.

[00467]

[00468] 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 $20\text{-}30 \times 10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 20×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 25×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 30×10^6 IU/mg for a 1 mg vial. In some embodiments, the IL-2 stock solution has a final concentration of $4\text{-}8 \times 10^6$ IU/mg of IL-2. In some embodiments, the IL-2 stock solution has a final concentration of $5\text{-}7 \times 10^6$ IU/mg of IL-2. In some embodiments, the IL-2 stock solution has a final concentration of 6×10^6 IU/mg of IL-2. In some embodiments, the IL-2 stock solution is

prepare as described in Example E. 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.

[00469] 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.

[00470] 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.

[00471] In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 μ g/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

[00472] In some embodiments, the cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some

embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 µg/mL and 100 µg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 µg/mL and 40 µg/mL.

[00473] In some embodiments, in addition to one or more TNFRSF agonists, the cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

[00474] 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 10cm² gas-permeable silicon bottom (for example, G-Rex10; Wilson Wolf Manufacturing, New Brighton, MN) (Fig. 1), each flask was loaded with 10×10^6 to 40×10^6 viable tumor digest cells or 5–30 tumor fragments in 10 to 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% CO₂ 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 to 3 days. In some embodiments, the CM is the CM1 described in the Examples, *see*, Example 1. In some embodiments, the first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the initial cell culture medium or the first cell culture medium comprises IL-2.

[00475] In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1, which can include those sometimes referred to as the pre-REP) process is shortened to 3 to 14 days, as discussed in the examples and figures. In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1, which can include those sometimes referred to as the pre-REP) is shortened to 7 to 14 days, as discussed in the Examples and shown in Figures 4 and 5, as well as including for example, an expansion as described in Step B of Figure 1. In some embodiments, the first expansion of Step B is shortened to 10-14 days. In some embodiments, the first expansion is

shortened to 11 days, as discussed in, for example, an expansion as described in Step B of Figure 1.

[00476] 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.

[00477] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first expansion, including for example during a Step B processes according to Figure 1, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the

first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 1 and as described herein.

[00478] In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 1) process is shortened to 3 to 14 days, as discussed in the examples and figures. In some embodiments, the first expansion of Step B is shortened to 7 to 14 days. In some embodiments, the first expansion of Step B is shortened to 10 to 14 days. In some embodiments, the first expansion is shortened to 11 days.

[00479] In some embodiments, the first expansion, for example, Step B according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

Step C: First Expansion to Second Expansion Transition

[00480] In some cases, the bulk TIL population obtained from the first expansion, including for example the TIL population obtained from for example, Step B as indicated in Figure 1, can be cryopreserved immediately, using the protocols discussed herein below. Alternatively, the TIL population obtained from the first expansion, referred to as the second TIL population, can be subjected to a second expansion (which can include expansions sometimes referred to as REP) and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the first TIL population (sometimes referred to as the bulk TIL population) or the second TIL population (which can in some embodiments include populations referred to as the REP TIL populations) can be subjected to genetic modifications for suitable treatments prior to expansion or after the first expansion and prior to the second expansion.

[00481] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1) are not stored and proceed directly to the second expansion. In some embodiments, the TILs obtained from the first expansion are not cryopreserved after the first expansion and prior to the second expansion. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11

days, 12 days, 13 days, or 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 7 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 14 days from when fragmentation occurs.

[00482] 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.

[00483] In some embodiments, the TILs are not stored after the first expansion and prior to the second expansion, and the TILs proceed directly to the second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 1). In some embodiments, the transition occurs in closed system, as described herein. In some embodiments, the TILs from the first expansion, the second population of TILs, proceeds directly into the second expansion with no transition period.

[00484] In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

Cytokines

[00485] 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.

[00486] 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.

Step D: Second Expansion

[00487] In some embodiments, the TIL cell population is expanded in number after harvest and initial bulk processing for example, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 1). This further expansion is referred to herein as the second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (REP; as well as processes as indicated in Step D of Figure 1). The second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container.

[00488] In some embodiments, the second expansion or second TIL expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 1) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the second TIL expansion can proceed for about 7 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 8 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 9 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 10 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 11 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 12 days to about 14 days. In some embodiments, the

second TIL expansion can proceed for about 13 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 14 days.

[00489] In an embodiment, the second expansion can be performed in a gas permeable container using the methods of the present disclosure (including for example, expansions referred to as REP; as well as processes as indicated in Step D of Figure 1). For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San Diego, CA, USA). TILs can be expanded to induce further stimulation of the TILs in vitro by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 μ M 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.

[00490] In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL,

between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or between 8000 IU/mL of IL-2.

[00491] In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 µg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

[00492] In some embodiments, the cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 µg/mL and 100 µg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 µg/mL and 40 µg/mL.

[00493] In some embodiments, in addition to one or more TNFRSF agonists, the cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

[00494] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the second expansion,

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

[00495] In some embodiments, the second expansion can be conducted in a supplemented cell culture medium comprising IL-2, OKT-3, antigen-presenting feeder cells, and optionally a TNFRSF agonist. In some embodiments, the second expansion occurs in a supplemented cell culture medium. In some embodiments, the supplemented cell culture medium comprises IL-2, OKT-3, and antigen-presenting feeder cells. In some embodiments, the second cell culture medium comprises IL-2, OKT-3, and antigen-presenting cells (APCs; also referred to as antigen-presenting feeder cells). In some embodiments, the second expansion occurs in a cell culture medium comprising IL-2, OKT-3, and antigen-presenting feeder cells (*i.e.*, antigen presenting cells).

[00496] 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.

[00497] 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.

[00498] 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.

[00499] 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.

[00500] 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.

[00501] 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 1×10^6 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% CO₂. 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 2.0×10^6 cells/mL.

[00502] In an embodiment, the second expansion (which can include expansions referred to as REP, as well as those referred to in Step D of Figure 1) may be performed in 500 mL capacity gas permeable flasks with 100 cm gas-permeable silicon bottoms (G-Rex 100, commercially available from Wilson Wolf Manufacturing Corporation, New Brighton, MN, USA), 5×10^6 or 10×10^6 TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3). The G-Rex 100 flasks may be incubated at 37°C in 5% CO₂. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and centrifuged at 1500 rpm ($491 \times g$) 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% CO₂ 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.

[00503] 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.

[00504] 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.

[00505] Optionally, a cell viability assay can be performed after the second expansion (including expansions referred to as the REP expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. In some embodiments, TIL samples can be counted and viability determined using a Cellometer K2 automated cell counter (Nexcelom Bioscience, Lawrence, MA). In some embodiments, viability is determined according to the standard Cellometer K2 Image Cytometer Automatic Cell Counter protocol.

[00506] 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 1×10^6 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% CO₂. 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 2.0×10^6 cells/mL.

[00507] In some embodiments, the second expansion (including expansions referred to as REP) are performed in 500 mL capacity flasks with 100 cm² gas-permeable silicon bottoms (G-Rex 100, Wilson Wolf) (Fig. 1), about 5×10^6 or 10×10^6 TIL are cultured with irradiated allogeneic PBMC at a ratio of 1 to 100 in 400 mL of 50/50 medium, supplemented with 3000 IU/mL of IL-2 and 30 ng/mL of anti-CD3. The G-Rex 100 flasks are incubated at 37°C in 5% CO₂. 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% CO₂ 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.

[00508] 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.*, TCR α/β).

[00509] 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.

[00510] In some embodiments, the second expansion, for example, Step D according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

Feeder Cells and Antigen Presenting Cells

[00511] In an embodiment, the second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 1, as well as those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation, *see, e.g.* “Isolation of mononuclear cells: Methodology and Application”, GE Life Sciences technical publication 18-1152-69-AE, *available at* https://us.vwr.com/assetsvc/asset/en_US/id/16286835/contents.

[00512] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

[00513] 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).

[00514] 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.

[00515] 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 to 60 ng/ml OKT3 antibody and 1000-6000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 10 to 50 ng/ml OKT3 antibody and 2000-5000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 20 to 40 ng/ml OKT3 antibody and 2000-4000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 25 to 35 ng/ml OKT3 antibody and 2500-3500 IU/ml IL-2.

[00516] 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.

[00517] In an embodiment, the second expansion procedures described herein require a ratio of about 2.5×10^9 feeder cells to about 100×10^6 TILs. In another embodiment, the second expansion procedures described herein require a ratio of about 2.5×10^9 feeder cells to about 50×10^6 TILs. In yet another embodiment, the second expansion procedures described herein require about 2.5×10^9 feeder cells to about 25×10^6 TILs.

[00518] 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.

[00519] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

[00520] In an embodiment, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

Cytokines

[00521] The TIL expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

[00522] 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.

Step E: Harvest TILs

[00523] After the second expansion step, cells can be harvested. In some embodiments the TILs are harvested after one, two, three, four or more expansion steps, for example as provided in Figure 1. In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 1.

[00524] 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.

[00525] 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.

[00526] In some embodiments, the harvest, for example, Step E according to Figure 1, is performed from a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

[00527] In some embodiments, Step E according to Figure 1, is performed according to the processes described in Example 7. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system, as described in Example 7, is employed.

[00528] In some embodiments, TILs are harvested according to the methods described in Example 7. In some embodiments, TILs between days 1 and 11 are harvested using the methods as described herein (referred to as the Day 11 TIL harvest in Example 7). In some embodiments, TILs between days 12 and 22 are harvested using the methods as described herein (referred to as the Day 22 TIL harvest in Example 7).

Step F: Final Formulation/ Transfer to Infusion Bag

[00529] After Steps A through E as provided in an exemplary order in Figure 1 and as outlined in detailed above and herein are complete, cells are transferred to a container for use in

administration to a patient. In some embodiments, once a therapeutically sufficient number of TILs are obtained using the expansion methods described above, they are transferred to a container for use in administration to a patient.

[00530] 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.

Anti-CD3 Antibodies as Optional Media Components

[00531] In some embodiments, the culture media used in expansion methods described herein (including those referred to as REP, see for example, Figure 1) also includes an anti-CD3 antibody. An anti-CD3 antibody in combination with IL-2 induces T cell activation and cell division in the TIL population. This effect can be seen with full length antibodies as well as Fab and F(ab')₂ fragments, with the former being generally preferred; *see, e.g.*, Tsoukas *et al.*, *J. Immunol.*, **1985**, 135, 1719, hereby incorporated by reference in its entirety.

[00532] 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 muromonab (including the embodiments shown in Table 1) is used (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA). Anti-CD3 antibodies also include the UHCT1 clone, also known as T3 and CD3ε. Other anti-CD3 antibodies include, for example, oteixizumab, teplizumab, and visilizumab.

4-1BB (CD137) Agonists as Optional Media Components

[00533] In an embodiment, the TNFRSF agonist is a 4-1BB (CD137) agonist. The 4-1BB agonist may be any 4-1BB binding molecule known in the art. The 4-1BB binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian 4-1BB.

The 4-1BB agonists or 4-1BB binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The 4-1BB agonist or 4-1BB binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to 4-1BB. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a humanized antibody. In some embodiments, 4-1BB agonists for use in the presently disclosed methods and compositions include anti-4-1BB antibodies, human anti-4-1BB antibodies, mouse anti-4-1BB antibodies, mammalian anti-4-1BB antibodies, monoclonal anti-4-1BB antibodies, polyclonal anti-4-1BB antibodies, chimeric anti-4-1BB antibodies, anti-4-1BB adnectins, anti-4-1BB domain antibodies, single chain anti-4-1BB fragments, heavy chain anti-4-1BB fragments, light chain anti-4-1BB fragments, anti-4-1BB fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. Agonistic anti-4-1BB antibodies are known to induce strong immune responses. Lee, *et al.*, *PLOS One*, **2013**, 8:e69677. In a preferred embodiment, the 4-1BB agonist is an agonistic, anti-4-1BB humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line). In an embodiment, the 4-1BB agonist is EU-101 (Eutilex Co. Ltd.), utomilumab, or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof. In a preferred embodiment, the 4-1BB agonist is utomilumab or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof.

[00534] 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, for example, in Gieffers, *et al.*, *Mol. Cancer Therapeutics*, **2013**, 12:2735-47.

[00535] 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.

[00536] 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 TLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR 60 TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC 120 CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPFPAPARE 180 PGHSPQIISF FLALTSTALL FLLFFLTFRF 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 CQPGTFCKRY NPVCKSCPPS TFSSIGGQPN 60 CNICRVCAGY FRFKKFCSS HNAECECIEG FHCLGPQCTR CEKDCRPGQE LTKQGCKTCS 120 LGTFNDQNGT GVCRPWTNCS LDGRSVLKTG TTEKDVVCGP PVVSFSPSTT ISVTPEGGPG 180 GHSLQVLTLF LALTSALLA LIFITLLFSV LKWIRKKFPH IFKQPFKKT GAAQEEDACS 240 CRCPQEEEGG GGGYEL 256

[00537] 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.

[00538] 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 1/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.

[00539] 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.

[00540] 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 IC₅₀ of about 2 nM or lower, or binds to human or murine 4-1BB with an IC₅₀ of about 1 nM or lower.

[00541] 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.

[00542] 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.

[00543] 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.

[00544] 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.

[00545] 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 IYPGDSYTN 60 SPSFQGGVTI SADKSISTAY LQWSSLKASD TAMYYCARGY GIFDYWGQGT LVTVSSASTK 120 GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVHTFP AVLQSSGLYS 180 LSSVTVFPSS NFGTQTYTCN VDHKPSNTKV DKTVERKCCV ECPPCPAPPV AGPSVFLFPP 240 KPKDTLMISR TPEVTCVVD VSHEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTFRVSV 300 LTVVHQDWLN GKEYKCKVSN KGLPAPIEKT ISKTKGQPRE PQVYTLPPSR EEMTKNQVSL 360 TCLVKGFYPS DIAVEWESNG QPENNYKTP PMLDSGSEFF LYSKLTVDKS RWQQGNVFSC 420 SVMHEALHNH YTQKSLSLSP G 441
SEQ ID NO:12 light chain for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER 60 FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGLAVFG GGTCLTVLGG PKAAPSVTLF 120 PPSSEELQAN KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTTPSKQS NNKYAASSYL 180 SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS 214
SEQ ID NO:13 heavy chain variable region for utomilumab	EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMG K IYPGDSYTN 60 YSPSFQGGVT ISADKSISTA YLQWSSLKAS DTAMYYCARG YGIFDYWGQ GTLTVVSS 118

SEQ ID NO:14 light chain variable region for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGLAVFG 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

[00546] 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.

[00547] 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.

[00548] 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.

[00549] 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.

[00550] 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 TCAVYGGSFSG GYVWSWIRQS PEKGLEWIGE INHGGYVTYN 60 PSLESRTVIS VDTSKNQFSL KLSSVTAADT AVYYCARDYG PGNYDWYFDL WGRGTLTVTS 120 SASTKGPSVF PLAPCSRSTS ESTAALGCLV KDYFPEPVTW SWNSGALTSG VHTFPAVLQS 180 SGLYSLSSVV TVPSSSLGSK TYTCNVDPKPK SNTKVDKRVK SKYGPCCPPC PAPEFLGGPS 240 VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGEVHNNAKT KPREEQFNST 300 YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT 360 KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE 420 GNVFSCSVMH EALHNHYTQK SLSLSLGK 448
SEQ ID NO:22 light chain for urelumab	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLIYD ASNRATGIPA 60 RFGSGSGSTD FTLTISSLEP EDFAVYYCQQ RSNWPPALTF CGGKVEIKR TVAAPS VFIF 120 PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYLSST 180 LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC 216
SEQ ID NO:23 variable heavy chain for urelumab	MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YVWSWIRQSP 60 EKGLEWIGEI NHGGYVTYNP SLESRTVISV DTSKNQFSLK LSSVTAADTA VYCARDYGP 120
SEQ ID NO:24 variable light chain for urelumab	MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP 60 GQAPRLIYD ASNRATGIPA RFGSGSGSTD FTLTISSLEP EDFAVYYCQQ 110
SEQ ID NO:25 heavy chain CDR1 for urelumab	GYYS 5
SEQ ID NO:26 heavy chain CDR2 for urelumab	EINHGGYVTY NPSLES 16
SEQ ID NO:27 heavy chain CDR3 for urelumab	DYGPNGYDWY 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	QQRSWPPAL T 11

[00551] 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-IgG1 (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 3E1), 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.

[00552] 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.

[00553] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein as depicted in Figure 25 as 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:

As shown in Figure 25 in structures I-A and I-B, the cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from *e.g.*, 4-1BBL or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second trivalent protein through IgG1-Fc (including CH3 and CH2 domains) and is then used to link two of the trivalent proteins together through disulfide bonds (small elongated ovals), stabilizing the structure and providing an agonist 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.

[00554] 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 VLNQDNLNGK EYKCKVSNKA LPAPIEKTIS 120 KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTTPPV 180 LDSGGSFFLY 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	GGPGSSSGSGS SDKTHTCPFC PAPE 24
SEQ ID NO:39 linker	GGPGSSSGSGS DKTHTCPFCP APE 23
SEQ ID NO:40 linker	GGPSSSGSDK THTCPFCPAP E 21
SEQ ID NO:41 linker	GGSSSSSSSS GSKTHTCPP CPAPE 25

[00555] 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 TNFRSF 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 60 CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK 120 CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE 180 WESNGQPENN YKTTTPVLDL DGSFFLYSKL TVDKSRWQQG NVFSCSVME ALHNHYTQKS 240 LSLSPG 246
SEQ ID NO:43 linker	SGSGSGSGSG S 11
SEQ ID NO:44 linker	SSSSSSSGSGS GS 12
SEQ ID NO:45 linker	SSSSSSSGSGS GSGSGS 16

[00556] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains selected from the group consisting of a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain of urelumab, a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table 4 or Table 5, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[00557] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:46. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a soluble 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:47.

[00558] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively, wherein the V_H and V_L domains are connected by a linker. In an

embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the V_H and V_L sequences given in Table 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 60 SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL 120 TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAAALA 180 LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVLHLTEA RARHAWQLTQ GATVLGLFRV 240 TPEIPAGLPS PRSE 254
SEQ ID NO:47 4-1BBL soluble domain	LRQGMFAQLV AQNVLLIDGP LSWYSDPGLA GVSLTGGLSY KEDTKELVVA KAGVYVFFQ 60 LELRRVVAGE GSGSVSLALH LQPLRSAAGA AALALTVDLP PASSEARNSA FGFQGRLLHL 120 SAGQRLGVHL HTEARARHAW QLTQGATVLG LFRVTPEIPA GLPSPRSE 168
SEQ ID NO:48 variable heavy chain for 4B4-1-1 version 1	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY 60 NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVS 118
SEQ ID NO:49 variable light chain for 4B4-1-1 version 1	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSIGGIPS 60 RFSGSGSGSD FTLINSINVEP EDVGYYCQD GHSFPPTFGG GTKLEIK 107
SEQ ID NO:50 variable heavy chain for 4B4-1-1 version 2	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY 60 NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVSA 119
SEQ ID NO:51 variable light chain for 4B4-1-1 version 2	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSIGGIPS 60 RFSGSGSGSD FTLINSINVEP EDVGYYCQD GHSFPPTFGG GTKLEIKR 108
SEQ ID NO:52 variable heavy chain for H39E3-2	MDWTWRILFL VAAATGAHSE VQLVESGGGL VQPGGSLRLS CAASGFTFSD YWMSWVRQAP 60 GKGLEWVADI KNDGSYTNYA PSLTNRTIS RDNANSLYL QMNSLRAEDT AVYYCARELT 120
SEQ ID NO:53 variable light chain for H39E3-2	MEAPAQLLFL LLLWLPDTTG DIVMTQSPDS LAVSLGERAT INCKSSQSL SSGNQKNYL 60 WYQQKPGQPP KLLIYYASTR QSGVPRFSG SSGTDFTLT ISSLQAEDVA 110

[00559] 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.

[00560] 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.

[00561] 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.

[00562] 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.

OX40 (CD134) Agonists as Optional Media Components

[00563] In an embodiment, the TNFRSF agonist is an OX40 (CD134) agonist. The OX40 agonist may be any OX40 binding molecule known in the art. The OX40 binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian OX40. The OX40 agonists or OX40 binding molecules may comprise an immunoglobulin heavy chain

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

[00564] 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, for example, in Gieffers, *et al.*, *Mol. Cancer Therapeutics*, **2013**, 12, 2735-47.

[00565] 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.

[00566] 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	60
	NTVCRPCGPG	FYNDVVSSKP	CKPCTWCNLR	SGSERKQLCT	ATQDTVCRCR	AGTQPLDSYK	120
	PGVDCAPCPP	GHFSPGDNQA	CKPWTNCTLA	GKHTLQPASN	SSDAICEDRD	PPATQPQETQ	180
	GPPARPITVQ	PTEAWPRTSQ	GPSTRPVEVP	GGRAVAAILG	LGLVLGLLGP	LAILLALYLL	240
	RRDQRLPPDA	HKPPGGGSEF	TPIQEEQADA	HSTLAKI			277
SEQ ID NO:55 murine OX40 (Mus musculus)	MYVWVQQPTA	LLLLGLTLGV	TARRLNCVKH	TYP SGHKCCR	ECQPGHGMVS	RCDHTRDTLC	60
	HPCETGFYNE	AVNYDTCKQC	TQCNHRSQSE	LKQNCPTPTQD	TVCRCRPGTQ	PRQDSGYKLG	120
	VDCVPCPPGH	FSPGNNQACK	PWTNCTLSGK	QTRHPASDSL	DAVCEDRSLL	ATLLWETQRP	180
	TFRPTTVQST	TVWPRTSELP	SPPTLVTPPEG	PAFAVLLGLG	LGLLAPLTVL	LALYLLRKAW	240
	RLPNTPKPCW	GNSFRTPIQE	EHTDAHFTLA	KI			272

[00567] 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.

[00568] 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.

[00569] 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.

[00570] 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.

[00571] 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.

[00572] 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.

[00573] 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.

[00574] 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.

[00575] 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 LVKPSQTL LSL TCAVYGGSF S SGYWNWIRKH PGKGLE YIGY ISYNGITYHN 60 PSLKSRLTIN RDTSKNQYSL QLNSTVPEDT AVYICARYKY DYDGGHAMDY WQGGTLVTVS 120 SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS 180 SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRV E PKSCDKTHTC PPCPAPELLG 240 GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY 300 NSTYRVVSVL TVLHQDWLNG KEYCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE 360 EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTTP VLDSDGSFFL YSKLTVDKSR 420 WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 451
SEQ ID NO:57 light chain for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS 60 RFGSGSGSTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKRTV AAPSVEIFPP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSSTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGE C 214
SEQ ID NO:58 heavy chain variable region for tavolixizumab	QVQLQESGPG LVKPSQTL LSL TCAVYGGSF S SGYWNWIRKH PGKGLE YIGY ISYNGITYHN 60 PSLKSRLTIN RDTSKNQYSL QLNSTVPEDT AVYICARYKY DYDGGHAMDY WQGGTLVT 118
SEQ ID NO:59 light chain variable region for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS 60 RFGSGSGSTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKR 108
SEQ ID NO:60 heavy chain CDR1 for tavolixizumab	GSFSSGYWN 9
SEQ ID NO:61 heavy chain CDR2 for tavolixizumab	YIGYISYNGI TYH 13
SEQ ID NO:62 heavy chain CDR3 for tavolixizumab	RYKYDYDGGH AMDY 14
SEQ ID NO:63 light chain CDR1 for tavolixizumab	QDISNYLN 8
SEQ ID NO:64 light chain CDR2 for tavolixizumab	LLIYYTSKLH S 11
SEQ ID NO:65 light chain CDR3 for tavolixizumab	QQGSALPW 8

[00576] 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.

[00577] 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.

[00578] 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.

[00579] 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.

[00580] 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 60 ADSVKGRFTI SRDIAKNSLY LQMNSLRDED TAVYYCARES GWYLFYWGQ GTLTVSSAS 120 TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL 180 YSLSSVTVTP SSNFGTQTYT CNVDHKPSNT KVDKTVKRC CVECPPCPAP PVAGPSVFLF 240 PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV QFNWYVDGVE VHNATKPRE EQFNSTFRVV 300 SVLTVVHQDW LNGKEYKCKV SNKGLPAPIE KTISKTKGQP REPQVYTLPP SREEMTKNQV 360 SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPMLDSGGS FFLYSKLTVD KSRWQQGNVF 420 SCSVMEALH NHYTQKSLSL SPGK 444
SEQ ID NO:67 light chain for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS 60 RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIKRTV AAPSVFIIPP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSLSTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEK 214
SEQ ID NO:68 heavy chain variable region for 11D4	EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY 60 ADSVKGRFTI SRDIAKNSLY LQMNSLRDED TAVYYCARES GWYLFYWGQ GTLTVSS 118
SEQ ID NO:69 light chain variable region for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS 60 RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIK 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	ESGWYLFY 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

[00581] 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.

[00582] 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.

[00583] 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.

[00584] 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.

[00585] 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	ISWNSGSGIGY	60
	ADSVKGRFTI	SRDNAKNSLY	LQMNSLRAED	TALYYCAKDQ	STADYYFFYYG	MDVWGQGGTTV	120
	TVSSASTKGP	SVFPLAPCSR	STSESTAALG	CLVKDYFPEP	VTVSWNSGAL	TSGVHTFFPAV	180
	LQSSGLYSLS	SVVTVPSSEF	GTQTYTCNVD	HKPSNTKVDK	TVERKCCVEC	PPCPAPPVAG	240
	PSVFLFPPKP	KDTLMISRTF	EVTCCVVDVS	HEDPEVQFNW	YVDGVEVHNA	KTKPREEQFN	300
	STFRVSVLT	VVHQDWLNGK	EYKCKVSNKG	LPAPIEKTIS	KTKGQPREPQ	VYTLPPSREE	360
	MTKNQVSLTC	LVKGFYPSDI	AVEWESNGQP	ENNYKTTPPM	LDSGGSFFLY	SKLTVDKSRW	420
	QQGNVFSCSV	MHEALHNHYT	QKSLSLSPGK				450
	EIVVTQSPAT	LSLSPGERAT	LSCRASQSVS	SYLAWYQQKP	GQAPRLLIYD	ASNRATGIPA	60
	RFSGSGSGTD	FTLTISSELP	EDFAVYYCQQ	RSNWPTFGQG	TKVEIKRTVA	APSVFIFPPS	120
	DEQLKSGTAS	VVCLLNNFYP	REAKVQWKVD	NALQSGNSQE	SVTEQDSKDS	TYSLSSTLTL	180
	SKADYEKHKV	YACEVTHQGL	SSPVTKSENR	GEC			213
SEQ ID NO:78 heavy chain variable region for 18D8	EVQLVESGGG	LVQPGRSLRL	SCAASGFTFD	DYAMHWVRQA	PGKGLEWVSG	ISWNSGSGIGY	60
	ADSVKGRFTI	SRDNAKNSLY	LQMNSLRAED	TALYYCAKDQ	STADYYFFYYG	MDVWGQGGTTV	120
	TVSS						124

SEQ ID NO:79 light chain variable region for 18D8	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLIYD ASNRATGIPA RFGSGSGTD 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

[00586] 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.

[00587] 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.

[00588] 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.

[00589] 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 PGKGLVLA INSDGGSTYY 60 PDTMERRFTI SRDPAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMTVSS 120
SEQ ID NO:87 light chain variable region for Hu119-122	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHY QKPGQAPRL LIYLASNLES 60 GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K 111
SEQ ID NO:88 heavy chain CDR1 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

[00590] 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.

[00591] 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.

[00592] 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.

[00593] 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 60 ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYIAMD YWGQGTITVTV 120 SS 122
SEQ ID NO:95 light chain variable region for Hu106-222	DIQMTQSPSS LSASVGDRVT ITCKASQDVS TAVAWYQQKPK GKAPKLLIYS ASYLYTGVPS 60 RFGSGSGSTD FTFITISLQP EDIATYYCQQ HYSTPRTFGQ GTKLEIK 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

[00594] 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.

[00595] 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.

[00596] 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.

[00597] 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 TNFRSF 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.

[00598] 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 19, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[00599] 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.

[00600] 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 KEGGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYS QEVNISLHYQ KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF CVL	60 120 180 183	
SEQ ID NO:103 OX40L soluble domain	SHRYPRIQSI KVQFTEYKKE KGFILTSQKE DEIMKVQNNNS VIINCDGFYL ISLKGYSQEQ VNISLHYQKD EEPLFQLKKV RSVNSLMVAS LTYKDKVYLN VTTDNTSLDD FHVNGGELIL IHQNPGEFCV L	60 120 131	
SEQ ID NO:104 OX40L soluble domain (alternative)	YPRIQSIKVQ FTEYKKEKGF ILTSQKEDEI MKVQNNNSVII NCDGFYLISL KGYFSQEVNI SLHYQKDEEP LFQLKKVRSV NSLMVASLTY KDKVYLNVT 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 WGQGLVTVTS	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 VVQPGSLRL SCAASGFTFS DYTMMNWVRQA PGKGLEWVSS ISGGSTYYAD SRKGRFTISR DNSKNTLYLQ MNNLRAEDTA VYYCARDRYF RQQNAFDYWG QGTTLVTVSSA	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 WGQGLVTVTS	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 LVHPPGSLRL SCAGSGFTFS SYAMHWVRQA PGKGLEWVSA IGTGGGTYYA DSVMGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCARYDN VMGLYWFYDW GQGTTLVTVSS	60 120	
SEQ ID NO:112 variable light chain for 023	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQ RSNWPPAFGG GTKVEIKR	60 108	

SEQ ID NO:113 heavy chain variable region	EVQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY INPYNDGTTY 60 NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCANY YGSSLSMDYWG QGTSVTVSS 119
SEQ ID NO:114 light chain variable region	DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS 60 RFGSGSGSTD YSLTISNLEQ EDIATYFCQQ GNTLPWTFGG GTKLEIKR 108
SEQ ID NO:115 heavy chain variable region	EVQLQQSGPE LVKPGASVKI SCKTSGYTFF DYTMMHWVKQS HGKSLEWIGG IYPNNGGSTY 60 NQNFKDKATL TVDKSSSTAY MEFRSLTSED SAVYYCARMG YHGPHLDFDV WGAGTTTVTS 120 P 121
SEQ ID NO:116 light chain variable region	DIVMTQSHKF MSTSLGDRVS ITCKASQDVG AAVAWYQQKP GQSPKLLIYW ASTRHTGVDP 60 RFTGGSGSTD FTLTISNVQS EDLTDYFCQQ YINYPLTFGG GTKLEIKR 108
SEQ ID NO:117 heavy chain variable region of humanized antibody	QIQLVQSGPE LKKPGETVKI SCKASGYTFT DYSMHWVKQA PGKGLKWMGW INTETGEPTY 60 ADDFKGRFAF SLETSASTAY LQINNKKED TATYFCANPY YDYVSYAM YWGHGTSVTV 120 SS 122
SEQ ID NO:118 heavy chain variable region of humanized antibody	QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY 60 ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYAM YWQGTTTVTV 120 SS 122
SEQ ID NO:119 light chain variable region of humanized antibody	DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD 60 RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK 107
SEQ ID NO:120 light chain variable region of humanized antibody	DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD 60 RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK 107
SEQ ID NO:121 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGESLKL SCESNEYEPF SHDMSWVRKT PEKRLLELVA INSDGGSTYY 60 PDTMERRFII SRDNTKTKLY LQMSSLRSED TALYYCARHY DDYYAWFAYW GQGLTVTVSA 120
SEQ ID NO:122 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGGSLRL SCAASEYEPF SHDMSWVRQA PGKGLLELVA INSDGGSTYY 60 PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMTVTVSS 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 SGTDFTLNIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K 111
SEQ ID NO:125 heavy chain variable region	MYLGLNYVFI VFLLNGVQSE VKLEESGGGL VQPGGSMKLS CAASGFTFSD AWMDWVRQSP 60 EKGLEWVAEI RSKANNHATY AESVNGRFT ISRDDSKSSV YLQMNSLRAE DTGIYYCTWG 120 EVFYFDYWGQ GTTTLTVSS 138
SEQ ID NO:126 light chain variable region	MRPSIQFLGL LLEWLHGAQC DIQMTQSPSS LSASLGKVT ITCKSSQDIN KYIAWYQHKP 60 GKGPRLLIHY TSTLQPGIPS RFGSGSGGRD YSFSISNLEP EDIATYYCLQ YDNLITFGAG 120 TKLELK 126

[00601] 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.

[00602] 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.

[00603] 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.

[00604] 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.

[00605] In an embodiment, the OX40 agonist is Creative Biolabs OX40 agonist monoclonal antibody MOM-18455, commercially available from Creative Biolabs, Inc., Shirley, NY, USA.

[00606] In an embodiment, the OX40 agonist is OX40 agonistic antibody clone Ber-ACT35, commercially available from BioLegend, Inc., San Diego, CA, USA.

Option Cell Viability Analyses

[00607] Optionally, a cell viability assay can be performed after the first expansion (sometimes referred to as the initial bulk expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels

dead cells and allows a viability assessment. Other assays for use in testing viability can include but are not limited to the Alamar blue assay; and the MTT assay.

Cell Counts, Viability, Flow Cytometry

[00608] In some embodiments, cell counts and/or viability are measured. The expression of markers such as but not limited CD3, CD4, CD8, and CD56, as well as any other disclosed or described herein, can be measured by flow cytometry with antibodies, for example but not limited to those commercially available from BD Bio-sciences (BD Biosciences, San Jose, CA) using a FACSCanto™ flow cytometer (BD Biosciences). The cells can be counted manually using a disposable c-chip hemocytometer (VWR, Batavia, IL) and viability can be assessed using any method known in the art, including but not limited to trypan blue staining. The cell viability can also be assayed based on US Application 15/863,634, "Processes for Production of Tumor Infiltrating Lymphocytes and Uses of Same in Immunotherapy," incorporated by reference herein in its entirety.

[00609] In some cases, the bulk TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to REP and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the bulk or REP TIL populations can be subjected to genetic modifications for suitable treatments.

[00610] According to the present disclosure, a method for assaying TILs for viability and/or further use in administration to a subject. In some embodiments, the method for assay tumor infiltrating lymphocytes (TILs) comprises:

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

- (iv) harvesting, washing, and cryopreserving the third population of TILs;
- (v) storing the cryopreserved TILs at a cryogenic temperature;
- (vi) thawing the third population of TILs to provide a thawed third population of TILs;
and
- (vii) performing an additional second expansion of a portion of the thawed third population of TILs by supplementing the cell culture medium of the third population with IL-2, OKT-3, and APCs for an additional expansion period (sometimes referred to as a reREP period) of at least 3 days, wherein the third expansion is performed to obtain a fourth population of TILs, wherein the number of TILs in the fourth population of TILs is compared to the number of TILs in the third population of TILs to obtain a ratio;
- (viii) determining based on the ratio in step (vii) whether the thawed population of TILs is suitable for administration to a patient;
- (ix) administering a therapeutically effective dosage of the thawed third population of TILs to the patient when the ratio of the number of TILs in the fourth population of TILs to the number of TILs in the third population of TILs is determined to be greater than 5:1 in step (viii).

[00611] In some embodiments, the TILs are assayed for viability after step (vii).

[00612] The present disclosure also provides further methods for assaying TILs. In some embodiments, the disclosure provides a method for assaying TILs comprising:

- (i) obtaining a portion of a first population of cryopreserved TILs;
- (ii) thawing the portion of the first population of cryopreserved TILs;
- (iii) performing a first expansion by culturing the portion of the first population of TILs in a cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs) for an additional expansion period (sometimes referred to as a reREP period) of at least 3 days, to produce a second population of TILs, wherein the portion from the first population of TILs is compared to the second population of TILs to obtain a ratio of the number of TILs, wherein the ratio of the number of TILs in the second population of TILs to the number of TILs in the portion of the first population of TILs

is greater than 5:1;

- (iv) determining based on the ratio in step (iii) whether the first population of TILs is suitable for use in therapeutic administration to a patient;
- (v) determining the first population of TILs is suitable for use in therapeutic administration when the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is determined to be greater than 5:1 in step (iv).

[00613] In some embodiments, the ratio of the number of TILs in the second population of TILs to the number of TILs in the portion of the first population of TILs is greater than 50:1.

[00614] In some embodiments, the method further comprises performing expansion of the entire first population of cryopreserved TILs from step (i) according to the methods as described in any of the embodiments provided herein.

[00615] In some embodiments, the method further comprises administering the entire first population of cryopreserved TILs from step (i) to the patient.

Cell Culture Media

[00616] In an embodiment, a method for expanding TILs, including those discussed above as well as exemplified in Figure 1, may include using about 5,000 mL to about 25,000 mL of cell medium, about 5,000 mL to about 10,000 mL of cell medium, or about 5,800 mL to about 8,700 mL of cell medium. In some embodiments, the media is a serum free medium. In some embodiments, the media in the first expansion is serum free. In some embodiments, the media in the second expansion is serum free. In some embodiments, the media in the first expansion and the second are both serum free. In an embodiment, expanding the number of TILs uses no more than one type of cell culture medium. Any suitable cell culture medium may be used, *e.g.*, AIM-V cell medium (L-glutamine, 50 μ M streptomycin sulfate, and 10 μ M 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.

[00617] In some embodiments, the culture medium used in the expansion processes disclosed herein is a serum-free medium or a defined medium. In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or a serum replacement. In some embodiments, the serum-free or defined medium is used to prevent and/or decrease experimental variation due in part to the lot-to-lot variation of serum-containing media.

[00618] In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or serum replacement. In some embodiments, the basal cell medium includes, but is not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-Cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (α MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00619] In some embodiments, the serum supplement or serum replacement includes, but is not limited to one or more of CTS™ OpTmizer T-Cell Expansion Serum Supplement, CTS™ Immune Cell Serum Replacement, one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more antibiotics, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^+ , Al^{3+} , Ba^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Ge^{4+} , Se^{4+} , Br, I, Mn^{2+} , P, Si^{4+} , V^{5+} , Mo^{6+} , Ni^{2+} , Rb^+ , Sn^{2+} and Zr^{4+} . In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[00620] In some embodiments, the CTS™ OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle

(BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (α MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00621] In some embodiments, the total serum replacement concentration (vol%) in the serum-free or defined medium is from about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% by volume of the total serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 3% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 5% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 10% of the total volume of the serum-free or defined medium.

[00622] In some embodiments, the serum-free or defined medium is CTST[™] OpTmizer[™] T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTST[™] OpTmizer[™] is useful in the present invention. CTST[™] OpTmizer[™] T-cell Expansion SFM is a combination of 1L CTST[™] OpTmizer[™] T-cell Expansion Basal Medium and 26 mL CTST[™] OpTmizer[™] T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTST[™] OpTmizer[™] T-cell Expansion SFM is supplemented with about 3% of the CTST[™] Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM.

[00623] In some embodiments, the defined medium is CTST[™] OpTmizer[™] T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTST[™] OpTmizer[™] is useful in the present invention. CTST[™] OpTmizer[™] T-cell Expansion SFM is a combination of 1L CTST[™] OpTmizer[™] T-cell Expansion Basal Medium and 26 mL CTST[™] OpTmizer[™] T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTST[™] OpTmizer[™] T-cell Expansion SFM is supplemented with about 3% of the CTST[™] Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTST[™] OpTmizer[™] T-cell Expansion SFM is supplemented with about 3% of the CTST[™] Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTST[™] OpTmizer[™] T-cell Expansion SFM is supplemented with about 3% of the CTST[™] Immune Cell Serum

Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 6000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTSTTMOpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and about 2mM glutamine, and further comprises about 6000 IU/mL of IL-2.

[00624] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

[00625] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM.

[00626] In some embodiments, the defined media described in International PCT Publication No. WO/1998/030679, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum-free culture. The serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L-hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^+ , Al^{3+} , Ba^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Ge^{4+} , Se^{4+} , Br, I, Mn^{2+} , P, Si^{4+} , V^{5+} , Mo^{6+} , Ni^{2+} , Rb^+ , Sn^{2+} and Zr^{4+} . In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (α MEM), Glasgow's

Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00627] In some embodiments, the concentration of glycine in the defined medium is in the range of from about 5-200 mg/L, the concentration of L- histidine is about 5-250 mg/L, the concentration of L-isoleucine is about 5-300 mg/L, the concentration of L-methionine is about 5-200 mg/L, the concentration of L-phenylalanine is about 5-400 mg/L, the concentration of L-proline is about 1-1000 mg/L, the concentration of L- hydroxyproline is about 1-45 mg/L, the concentration of L-serine is about 1-250 mg/L, the concentration of L-threonine is about 10-500 mg/L, the concentration of L-tryptophan is about 2-110 mg/L, the concentration of L-tyrosine is about 3-175 mg/L, the concentration of L-valine is about 5-500 mg/L, the concentration of thiamine is about 1-20 mg/L, the concentration of reduced glutathione is about 1-20 mg/L, the concentration of L-ascorbic acid-2-phosphate is about 1-200 mg/L, the concentration of iron saturated transferrin is about 1-50 mg/L, the concentration of insulin is about 1-100 mg/L, the concentration of sodium selenite is about 0.000001-0.0001 mg/L, and the concentration of albumin (e.g., AlbuMAX® I) is about 5000-50,000 mg/L.

[00628] In some embodiments, the non-trace element moiety ingredients in the defined medium are present in the concentration ranges listed in the column under the heading "Concentration Range in 1X Medium" in Table A below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading "A Preferred Embodiment of the 1X Medium" in Table A below. In other embodiments, the defined medium is a basal cell medium comprising a serum free supplement. In some of these embodiments, the serum free supplement comprises non-trace moiety ingredients of the type and in the concentrations listed in the column under the heading "A Preferred Embodiment in Supplement" in Table A below.

Table A: Concentrations of Non-Trace Element Moiety Ingredients

Ingredient	A preferred embodiment in supplement (mg/L) (About)	Concentration range in 1X medium (mg/L) (About)	A preferred embodiment in 1X medium (mg/L) (About)
Glycine	150	5-200	53
L-Histidine	940	5-250	183
L-Isoleucine	3400	5-300	615

L-Methionine	90	5-200	44
L-Phenylalanine	1800	5-400	336
L-Proline	4000	1-1000	600
L-Hydroxyproline	100	1-45	15
L-Serine	800	1-250	162
L-Threonine	2200	10-500	425
L-Tryptophan	440	2-110	82
L-Tyrosine	77	3-175	84
L-Valine	2400	5-500	454
Thiamine	33	1-20	9
Reduced Glutathione	10	1-20	1.5
Ascorbic Acid-2-PO ₄ (Mg Salt)	330	1-200	50
Transferrin (iron saturated)	55	1-50	8
Insulin	100	1-100	10
Sodium Selenite	0.07	0.000001-0.0001	0.00001
AlbuMAX [®] I	83,000	5000-50,000	12,500

[00629] In some embodiments, the osmolality of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolality is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μ M), 2-mercaptoethanol (final concentration of about 100 μ M).

[00630] In some embodiments, the defined media described in Smith, *et al.*, “Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement,” *Clin Transl Immunology*, 4(1) 2015 (doi: 10.1038/cti.2014.31) are useful in the present invention. Briefly, RPMI or CTS[™] OpTmizer[™] was used as the basal cell medium, and supplemented with either 0, 2%, 5%, or 10% CTS[™] Immune Cell Serum Replacement.

[00631] In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME or β ME; also known as 2-mercaptoethanol, CAS 60-24-2).

[00632] 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, for example, about 11 days. In some embodiments pre-REP is about 7 to 14 days, for example, about 11 days. In some embodiments, REP is about 7 to 14 days, for example, about 11 days.

[00633] 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, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the OMNI C3[®] cell culture bag (Lampire Biological Laboratories). In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the EXP-PAK[™] Cell Expansion Bio-Containers (Charter Medical). In an embodiment, the cell expansion system for expanding TILs uses the same gas permeable containers for each expansion step. In an embodiment, the cell expansion system for expanding TILs uses different gas permeable containers for each expansion step. 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.

[00634] In an embodiment, TILs can be expanded in G-Rex flasks (commercially available from Wilson Wolf Manufacturing). Such embodiments allow for cell populations to expand from about 5×10^5 cells/cm² to between 10×10^6 and 30×10^6 cells/cm². 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.

Optional Genetic Engineering of TILs

[00635] In some embodiments, the TILs are optionally genetically engineered to include additional functionalities, including, but not limited to, a high-affinity T cell receptor (TCR), for example, a TCR targeted at a tumor-associated antigen such as MAGE-1, HER2, or NY-ESO-1, or a chimeric antigen receptor (CAR) which binds to a tumor-associated cell surface molecule (*e.g.*, mesothelin) or lineage-restricted cell surface molecule (*e.g.*, CD19).

Closed Systems for TIL Manufacturing

[00636] 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.

[00637] Closed systems are 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/Guid>

ances/Blood/ucm076779.htm.

[00638] Sterile connecting devices (STCDs) produce sterile welds between two pieces of compatible tubing. This procedure permits sterile connection of a variety of containers and tube diameters. In some embodiments, the closed systems include luer lock and heat sealed systems as described in for example, Example 4. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in Example 6 is employed. In some embodiments, the TILs are formulated into a final product formulation container according to the method described in Example 4, section “Final Formulation and Fill”.

[00639] 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.

[00640] 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%.

[00641] The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

[00642] 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.

[00643] 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.

[00644] 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.

Optional Cryopreservation of TILs

[00645] As discussed above, and exemplified in Steps A through E as provided in Figure 1, cryopreservation can occur at numerous points throughout the TIL expansion process, including at the final stage of the process after TIL harvesting for preservation of the therapeutic product. In some embodiments, the expanded population of TILs after the second expansion (as provided for example, according to Step D of Figure 1) can be cryopreserved. Cryopreservation can be

generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80°C, with optional transfer to gaseous nitrogen freezers for cryopreservation. *See Sadeghi, et al., Acta Oncologica, 2013, 52, 978-986.* In some embodiments, the TILs are cryopreserved in 5% DMSO. In some embodiments, the TILs are cryopreserved in cell culture media plus 5% DMSO. In some embodiments, the TILs are cryopreserved according to the methods provided in Examples 6 and 7.

[00646] 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.

[00647] 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 1. In some embodiments, the TILs are cryopreserved in the infusion bag. In some embodiments, the TILs are cryopreserved prior to placement in an infusion bag. In some embodiments, the TILs are cryopreserved and not placed in an infusion bag. In some embodiments, cryopreservation is performed using a cryopreservation medium. In some embodiments, the cryopreservation media contains dimethylsulfoxide (DMSO). This is generally accomplished by putting the TIL population into a freezing solution, *e.g.* 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80°C, with optional transfer to gaseous nitrogen freezers for cryopreservation. *See, Sadeghi, et al., Acta Oncologica, 2013, 52, 978-986.*

[00648] 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.

[00649] 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.

[00650] 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.*

[00651] 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.

[00652] 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.

Pharmaceutical Compositions, Dosages, and Dosing Regimens

[00653] 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.

[00654] 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 if 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.

[00655] 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} .

[00656] 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.

[00657] 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%, 1.25%, 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.

[00658] 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.

[00659] 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.

[00660] 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.

[00661] 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 g, 3 g, 3.5 g, 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.

[00662] 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.

[00663] 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.

[00664] 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} .

[00665] 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.

[00666] 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.

[00667] 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.

Methods of Treating Patients

[00668] Methods of treatment begin with the initial TIL collection and culture of TILs. Such methods have been both described in the art by, for example, Jin *et al.*, *J. Immunotherapy*, **2012**, 35(3):283-292, incorporated by reference herein in its entirety. Embodiments of methods of treatment are described throughout the sections below, including the Examples.

[00669] The expanded TILs produced according the methods described herein, including for example as described in Steps A through F above or according to Steps A through F above (also as shown, for example, in Figure 1) find particular use in the treatment of patients with cancer (for example, as described in Goff, *et al.*, *J. Clinical Oncology*, **2016**, 34(20):2389-239, as well as the supplemental content; incorporated by reference herein in its entirety. In some embodiments, TIL were grown from resected deposits of metastatic melanoma as previously described (*see*, Dudley, *et al.*, *J Immunother.*, **2003**, 26:332-342; incorporated by reference herein in its entirety). Fresh tumor can be dissected under sterile conditions. A representative sample can be collected for formal pathologic analysis. Single fragments of 2 mm³ to 3 mm³ may be used. In some embodiments, 5, 10, 15, 20, 25 or 30 samples per patient are obtained. In some embodiments, 20, 25, or 30 samples per patient are obtained. In some embodiments, 20, 22, 24, 26, or 28 samples per patient are obtained. In some embodiments, 24 samples per patient are obtained. Samples can be placed in individual wells of a 24-well plate, maintained in growth media with high-dose IL-2 (6,000 IU/mL), and monitored for destruction of tumor and/or proliferation of TIL. Any tumor with viable cells remaining after processing can be enzymatically digested into a single cell suspension and cryopreserved, as described herein.

[00670] In some embodiments, successfully grown TIL can be sampled for phenotype analysis (CD3, CD4, CD8, and CD56) and tested against autologous tumor when available. TIL can be considered reactive if overnight coculture yielded interferon-gamma (IFN- γ) levels > 200 pg/mL and twice background. (Goff, *et al.*, *J Immunother.*, **2010**, 33:840-847; incorporated by reference herein in its entirety). In some embodiments, cultures with evidence of autologous reactivity or sufficient growth patterns can be selected for a second expansion (for example, a second expansion as provided in according to Step D of Figure 1), including second expansions that are sometimes referred to as rapid expansion (REP). In some embodiments, expanded TILs with high autologous reactivity (for example, high proliferation during a second expansion), are selected for an additional second expansion. In some embodiments, TILs with high autologous reactivity (for example, high proliferation during second expansion as provided in Step D of Figure 1), are selected for an additional second expansion according to Step D of Figure 1.

[00671] In some embodiments, the patient is not moved directly to ACT (adoptive cell transfer), for example, in some embodiments, after tumor harvesting and/or a first expansion, the cells are not utilized immediately. In such embodiments, TILs can be cryopreserved and thawed 2 days before administration to a patient. In such embodiments, TILs can be cryopreserved and thawed 1 day before administration to a patient. In some embodiments, the TILs can be cryopreserved and thawed immediately before the administration to a patient.

[00672] Cell phenotypes of cryopreserved samples of infusion bag TIL can be analyzed by flow cytometry (e.g., FlowJo) for surface markers CD3, CD4, CD8, CCR7, and CD45RA (BD BioSciences), as well as by any of the methods described herein. Serum cytokines were measured by using standard enzyme-linked immunosorbent assay techniques. A rise in serum IFN- γ was defined as >100 pg/mL and greater than 4 3 baseline levels.

[00673] In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 1, provide for a surprising improvement in clinical efficacy of the TILs. In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 1, exhibit increased clinical efficacy as compared to TILs produced by methods other than those described herein, including for example, methods other than those exemplified in Figure 1. In some embodiments, the methods other than those described herein include methods referred to as process 1C and/or Generation 1 (Gen 1). In some embodiments,

the increased efficacy is measured by DCR, ORR, and/or other clinical responses. In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 1, exhibit a similar time to response and safety profile compared to TILs produced by methods other than those described herein, including for example, methods other than those exemplified in Figure 1, for example the Gen 1 process.

[00674] In some embodiments, IFN-gamma (IFN- γ) is indicative of treatment efficacy and/or increased clinical efficacy. In some embodiments, IFN- γ in the blood of subjects treated with TILs is indicative of active TILs. In some embodiments, a potency assay for IFN- γ production is employed. IFN- γ production is another measure of cytotoxic potential. IFN- γ production can be measured by determining the levels of the cytokine IFN- γ in the blood, serum, or TILs *ex vivo* of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1. In some embodiments, an increase in IFN- γ is indicative of treatment efficacy in a patient treated with the TILs produced by the methods of the present invention. In some embodiments, IFN- γ is increased one-fold, two-fold, three-fold, four-fold, or five-fold or more as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased one-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased three-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased four-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased five-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example,

methods other than those embodied in Figure 1. In some embodiments, IFN- γ is measured using a Quantikine ELISA kit. In some embodiments, IFN- γ is measured in TILs *ex vivo* of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1. In some embodiments, IFN- γ is measured in blood of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1. In some embodiments, IFN- γ is measured in TILs serum of a subject treated with TILs prepared by the methods of the present invention, including those as described for example in Figure 1.

[00675] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 1, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 1, such as for example, methods referred to as process 1C methods. In some embodiments, significantly improved polyclonality and/or increased polyclonality is indicative of treatment efficacy and/or increased clinical efficacy. In some embodiments, polyclonality refers to the T-cell repertoire diversity. In some embodiments, an increase in polyclonality can be indicative of treatment efficacy with regard to administration of the TILs produced by the methods of the present invention. In some embodiments, polyclonality is increased one-fold, two-fold, ten-fold, 100-fold, 500-fold, or 1000-fold as compared to TILs prepared using methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased one-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased ten-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 100-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 500-fold as

compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 1000-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1.

[00676] Measures of efficacy can include the disease control rate (DCR) as well as overall response rate (ORR), as known in the art as well as described herein.

Methods of Treating Cancer and Other Diseases

[00677] 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.

[00678] 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, endometrial cancer, thyroid cancer, colorectal 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.

[00679] 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/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 present disclosure, the patient receives an intravenous infusion of IL-2 intravenously at

720,000 IU/kg every 8 hours to physiologic tolerance.

[00680] Efficacy of the compounds and combinations of compounds 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.

[00681] 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 1. 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 subjects treated with TILs prepared using methods referred to as process 1C, as exemplified in Figure 5 and/or Figure 6. In some embodiments, an increase in IFN- γ is indicative of treatment efficacy in a patient treated with the TILs produced by the methods of the present invention. In some embodiments, IFN- γ is increased one-fold, two-fold, three-fold, four-fold, or five-fold or more as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased one-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example,

methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased three-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased four-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ secretion is increased five-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, IFN- γ is measured using a Quantikine ELISA kit. In some embodiments, IFN- γ is measured 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.

[00682] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 1, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 1, such as for example, methods referred to as process 1C methods. In some embodiments, significantly improved polyclonality and/or increased polyclonality is indicative of treatment efficacy and/or increased clinical efficacy for cancer treatment. In some embodiments, polyclonality refers to the T-cell repertoire diversity. In some embodiments, an increase in polyclonality can be indicative of treatment efficacy with regard to administration of the TILs produced by the methods of the present invention. In some embodiments, polyclonality is increased one-fold, two-fold, ten-fold, 100-fold, 500-fold, or 1000-fold as compared to TILs prepared using methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased one-fold as compared to an untreated patient and/or as

compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased two-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased ten-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 100-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 500-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, polyclonality is increased 1000-fold as compared to an untreated patient and/or as compared to a patient treated with TILs prepared using other methods than those provided herein including for example, methods other than those embodied in Figure 1.

[00683] In some embodiments, the invention provides a method of treating cancer in a patient comprising administering to the patient a therapeutically effective dosage of TILs prepared using any of the foregoing methods of expanding TILs from frozen tumor tissue. In some embodiments, the cancer is a solid tumor cancer. In some embodiments, the solid tumor cancer is selected from the group consisting of melanoma, ovarian cancer, endometrial cancer, thyroid cancer, colorectal 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 cancer is a hematological malignancy. In some embodiments, the hematological malignancy 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.

[00684] In some embodiments, the invention provides a pharmaceutical composition comprising TILs prepared using any of the foregoing methods of expanding TILs from frozen

tumor tissue and a pharmaceutically acceptable carrier.

[00685] In some embodiments, the invention provides a pharmaceutical composition comprising TILs prepared using any of the foregoing methods of expanding TILs from frozen tumor tissue and a pharmaceutically acceptable carrier for use in a method of treating cancer in a patient, the method comprising administering to the patient a therapeutically effective dosage of the pharmaceutical composition. In some embodiments, the cancer is a solid tumor cancer. In some embodiments, the solid tumor cancer is selected from the group consisting of melanoma, ovarian cancer, endometrial cancer, thyroid cancer, colorectal 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 cancer is a hematological malignancy. In some embodiments, the hematological malignancy 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.

[00686] In some embodiments, the invention provides the use of a pharmaceutical composition comprising TILs prepared using any of the foregoing methods of expanding TILs from frozen tumor tissue and a pharmaceutically acceptable carrier in a method of treating cancer in a patient, the method comprising administering to the patient a therapeutically effective dosage of the pharmaceutical composition. In some embodiments, the cancer is a solid tumor cancer. In some embodiments, the solid tumor cancer is selected from the group consisting of melanoma, ovarian cancer, endometrial cancer, thyroid cancer, colorectal 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 cancer is a hematological malignancy. In some embodiments, the hematological malignancy 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.

Methods of co-administration

[00687] In some embodiments, the TILs produced as described herein, including for example TILs derived from a method described in Steps A through F of Figure 1, 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 (Tesar, 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-L1 are disclosed in U.S. Patent No. 7,943,743, herein incorporated by reference. It will be understood by one of ordinary skill that any antibody which binds to PD-1 or PD-L1, disrupts the PD-1/PD-L1 interaction, and stimulates an anti-tumor immune response, are 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 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 anti-PD-1 when the patient has refractory melanoma.

In some embodiments, the patient is administered TILs in combination with anti-PD-1 when the patient has non-small-cell lung carcinoma (NSCLC).

Optional Lymphodepletion Preconditioning of Patients

[00688] 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/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 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.

[00689] 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.

[00690] 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.

[00691] In some embodiments, the fludarabine is administered at a concentration of 0.5 µg/mL - 10 µg/mL fludarabine. In some embodiments, the fludarabine is administered at a concentration

of 1 µg/mL fludarabine. In some embodiments, the fludarabine treatment is administered for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days or more. In some embodiments, the fludarabine is administered at a dosage of 10 mg/kg/day, 15 mg/kg/day, 20 mg/kg/day, 25 mg/kg/day, 30 mg/kg/day, 35 mg/kg/day, 40 mg/kg/day, or 45 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 2-7 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4-5 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4-5 days at 25 mg/kg/day.

[00692] 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/m²/day, 150 mg/m²/day, 175 mg/m²/day, 200 mg/m²/day, 225 mg/m²/day, 250 mg/m²/day, 275 mg/m²/day, or 300 mg/m²/day. In some embodiments, the cyclophosphamide is administered intravenously (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/m²/day i.v. In some embodiments, the cyclophosphamide treatment is administered for 4 days at 250 mg/m²/day i.v.

[00693] 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/m²/day i.v. and cyclophosphamide is administered at 250 mg/m²/day i.v. over 4 days.

[00694] In an embodiment, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m²/day for two days followed by administration of fludarabine at a dose of 25 mg/m²/day for five days.

IL-2 Regimens

[00695] 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.

[00696] 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/m² administered intravenously over 6 hours, followed by 18×10^6 IU/m² administered intravenously over 12 hours, followed by 18×10^6 IU/m² administered intravenously over 24 hrs, followed by 4.5×10^6 IU/m² 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/m² on day 1, 9,000,000 IU/m² on day 2, and 4,500,000 IU/m² on days 3 and 4.

[00697] 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.

Adoptive Cell Transfer

[00698] 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 in the TIL manufacturing process herein, including using a tumor cryopreservation and thawing step. In some embodiments, the TILs are prepared, for example, according to a method as described in Figure 1. 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.

Combinations with PD-1 and PD-L1 Inhibitors

[00699] Programmed death 1 (PD-1) is a 288-amino acid transmembrane immunoreceptor protein expressed by T cells, B cells, natural killer (NK) T cells, activated monocytes, and dendritic cells. PD-1, which is also known as CD279, belongs to the CD28 family, and in humans is encoded by the *Pdcd1* gene on chromosome 2. PD-1 consists of one immunoglobulin (Ig) superfamily domain, a transmembrane region, and an intracellular domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 and its ligands (PD-L1 and PD-L2) are known to play a key role in immune tolerance, as described in Keir, *et al.*, *Annu. Rev. Immunol.* **2008**, 26:677-704. PD-1 provides inhibitory signals that negatively regulate T cell immune responses. PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) are expressed on tumor cells and stromal cells, which may be encountered by activated T cells expressing PD-1, leading to immunosuppression of the T cells. PD-L1 is a 290 amino acid transmembrane protein encoded by the *Cd274* gene on human chromosome 9. Blocking the interaction between PD-1 and its ligands PD-L1 and PD-L2 by use of a PD-1 inhibitor, a PD-L1 inhibitor, and/or a PD-L2 inhibitor can overcome immune resistance, as demonstrated in recent clinical studies, such as that described in Topalian, *et al.*, *N. Eng. J. Med.* **2012**, 366:2443-54. PD-L1 is expressed on many tumor cell lines, while PD-L2 is expressed mostly on dendritic cells and a few tumor lines. In addition to T cells (which inducibly express PD-1 after activation), PD-1 is also expressed on B cells, natural killer cells, macrophages, activated monocytes, and dendritic cells.

[00700] The methods, compositions, and combinations of TILs and TNFRSF agonists described herein may also be further combined with programmed death-1 (PD-1), programmed death ligand 1 (PD-L1), and/or programmed death ligand 2 (PD-L2) binding antibodies, antagonists, or inhibitors (*i.e.*, blockers). PD-1, PD-L1, and/or PD-L2 inhibitors may be used in cell culture in conjunction with the TNFRSF agonists described herein during the pre-REP or REP stages of TIL expansion. PD-1, PD-L1, and/or PD-L2 inhibitors may also be used in conjunction with TNFRSF agonists prior to surgical resection of tumor, or during or after infusion of TILs. For example, suitable methods of using PD-1/PD-L1 inhibitors in conjunction with agonistic GITR antibodies and compositions comprising PD-1/PD-L1 antagonists and GITR agonists are described in International Patent Application Publication No. WO 2015/026684 A1, the disclosures of which are incorporated by reference herein.

[00701] In an embodiment, the PD-1 inhibitor may be any PD-1 inhibitor or PD-1 blocker known in the art. In particular, it is one of the PD-1 inhibitors or blockers described in more detail in the following paragraphs. The terms “inhibitor,” “antagonist,” and “blocker” are used interchangeably herein in reference to PD-1 inhibitors. For avoidance of doubt, references herein to a PD-1 inhibitor that is an antibody may refer to a compound or antigen-binding fragments, variants, conjugates, or biosimilars thereof. For avoidance of doubt, references herein to a PD-1 inhibitor may also refer to a small molecule compound or a pharmaceutically acceptable salt, ester, solvate, hydrate, cocrystal, or prodrug thereof.

[00702] In some embodiments, the compositions and methods described herein include a PD-1 inhibitor. In some embodiments, the PD-1 inhibitor is a small molecule. In a preferred embodiment, the PD-1 inhibitor is an antibody (*i.e.*, an anti-PD-1 antibody), a fragment thereof, including Fab fragments, or a single-chain variable fragment (scFv) thereof. In some embodiments the PD-1 inhibitor is a polyclonal antibody. In a preferred embodiment, the PD-1 inhibitor is a monoclonal antibody. In some embodiments, the PD-1 inhibitor competes for binding with PD-1, and/or binds to an epitope on PD-1. In an embodiment, the antibody competes for binding with PD-1, and/or binds to an epitope on PD-1.

[00703] In some embodiments, the compositions and methods described include a PD-1 inhibitor that binds human PD-1 with a K_D of about 100 pM or lower, binds human PD-1 with a K_D of about 90 pM or lower, binds human PD-1 with a K_D of about 80 pM or lower, binds

human PD-1 with a K_D of about 70 pM or lower, binds human PD-1 with a K_D of about 60 pM or lower, binds human PD-1 with a K_D of about 50 pM or lower, binds human PD-1 with a K_D of about 40 pM or lower, binds human PD-1 with a K_D of about 30 pM or lower, binds human PD-1 with a K_D of about 20 pM or lower, binds human PD-1 with a K_D of about 10 pM or lower, or binds human PD-1 with a K_D of about 1 pM or lower.

[00704] In some embodiments, the compositions and methods described include a PD-1 inhibitor that binds to human PD-1 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human PD-1 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human PD-1 with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human PD-1 with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human PD-1 with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human PD-1 with a k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human PD-1 with a k_{assoc} of about 1×10^6 1/M·s or faster.

[00705] In some embodiments, the compositions and methods described include a PD-1 inhibitor that binds to human PD-1 with a k_{dissoc} of about 2×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human PD-1 with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human PD-1 with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[00706] In some embodiments, the compositions and methods described include a PD-1 inhibitor that blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 10 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 9 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 8 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 7 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 6 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 5 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human

PD-1 with an IC₅₀ of about 4 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 3 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 2 nM or lower, or blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 1 nM or lower.

[00707] In an embodiment, the PD-1 inhibitor is nivolumab (commercially available as OPDIVO from Bristol-Myers Squibb Co.), or biosimilars, antigen-binding fragments, conjugates, or variants thereof. Nivolumab is a fully human IgG4 antibody blocking the PD-1 receptor. In an embodiment, the anti-PD-1 antibody is an immunoglobulin G4 kappa, anti-(human CD274) antibody. Nivolumab is assigned Chemical Abstracts Service (CAS) registry number 946414-94-4 and is also known as 5C4, BMS-936558, MDX-1106, and ONO-4538. The preparation and properties of nivolumab are described in U.S. Patent No. 8,008,449 and International Patent Publication No. WO 2006/121168, the disclosures of which are incorporated by reference herein. The clinical safety and efficacy of nivolumab in various forms of cancer has been described in Wang, *et al.*, *Cancer Immunol Res.* **2014**, 2:846-56; Page, *et al.*, *Ann. Rev. Med.*, **2014**, 65, 185-202; and Weber, *et al.*, *J. Clin. Oncology*, **2013**, 31:4311-4318, the disclosures of which are incorporated by reference herein. The amino acid sequences of nivolumab are set forth in Table 16. Nivolumab has intra-heavy chain disulfide linkages at 22-96, 140-196, 254-314, 360-418, 22"-96", 140"-196", 254"-314", and 360"-418"; intra-light chain disulfide linkages at 23'-88', 134'-194', 23'''-88''', and 134'''-194'''; inter-heavy-light chain disulfide linkages at 127-214', 127"-214'", inter-heavy-heavy chain disulfide linkages at 219-219" and 222-222"; and N-glycosylation sites (H CH₂ 84.4) at 290, 290".

[00708] In an embodiment, a PD-1 inhibitor comprises a heavy chain given by SEQ ID NO:127 and a light chain given by SEQ ID NO:128. In an embodiment, a PD-1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:127 and SEQ ID NO:128, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:127 and SEQ ID NO:128, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:127 and SEQ ID NO:128, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains

that are each at least 97% identical to the sequences shown in SEQ ID NO:127 and SEQ ID NO:128, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:127 and SEQ ID NO:128, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:127 and SEQ ID NO:128, respectively.

[00709] In an embodiment, the PD-1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of nivolumab. In an embodiment, the PD-1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:129, and the PD-1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:130, and conservative amino acid substitutions thereof. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:129 and SEQ ID NO:130, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:129 and SEQ ID NO:130, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:129 and SEQ ID NO:130, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:129 and SEQ ID NO:130, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:129 and SEQ ID NO:130, respectively.

[00710] In an embodiment, a PD-1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:131, SEQ ID NO:132, and SEQ ID NO:133, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:134, SEQ ID NO:135, and SEQ ID NO:136, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-1 as any of the aforementioned antibodies.

[00711] In an embodiment, the PD-1 inhibitor is an anti-PD-1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to nivolumab. In an embodiment, the

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

TABLE 16. Amino acid sequences for PD-1 inhibitors related to nivolumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:127 nivolumab heavy chain	QVQLVESGGG VVQPGRSRLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY 60 ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTLLVT VSSASTKGPS 120 VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS 180 VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPCCP PCPAPEFLGG PSVFLFPPKP 240 KDTLMISRTF EVTCVVVDVS QEDPEVQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT 300 VLHQDWLNGK EYKCKVSNKG LPSSIEKTIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLTC 360 LVKGFYPSDI AVEWESNGQP ENNYKTTTPPV LDSDGSFFLY SRLTVDKSRW QEGNVFSCSV 420 MHEALHNHYT QKSLSLSLGK 440
SEQ ID NO:128 nivolumab light chain	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA 60 RFSGSGSGTD FTLTISSLEP EDFAVYYCQK SSNWPRTFGQ GTKVEIKRTV AAPSVFIFFP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC 214
SEQ ID NO:129 nivolumab variable heavy chain	QVQLVESGGG VVQPGRSRLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRYY 60 ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTLLVT VSS 113
SEQ ID NO:130	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA 60 RFSGSGSGTD FTLTISSLEP EDFAVYYCQK SSNWPRTFGQ GTKVEIK 107

Identifier	Sequence (One-Letter Amino Acid Symbols)	
nivolumab variable light chain		
SEQ ID NO:131 nivolumab heavy chain CDR1	NSGMH	5
SEQ ID NO:132 nivolumab heavy chain CDR2	VIWYDGSKRY YADSVKG	17
SEQ ID NO:133 nivolumab heavy chain CDR3	NDDY	4
SEQ ID NO:134 nivolumab light chain CDR1	RASQSVSSYL A	11
SEQ ID NO:135 nivolumab light chain CDR2	DASNRAT	7
SEQ ID NO:136 nivolumab light chain CDR3	QQSSNWPRT	9

[00712] In another embodiment, the PD-1 inhibitor comprises pembrolizumab (commercially available as KEYTRUDA from Merck & Co., Inc., Kenilworth, NJ, USA), or antigen-binding fragments, conjugates, or variants thereof. Pembrolizumab is assigned CAS registry number 1374853-91-4 and is also known as lambrolizumab, MK-3475, and SCH-900475.

Pembrolizumab has an immunoglobulin G4, anti-(human protein PDCD1 (programmed cell death 1)) (human-Mus musculus monoclonal heavy chain), disulfide with human-Mus musculus monoclonal light chain, dimer structure. The structure of pembrolizumab may also be described as immunoglobulin G4, anti-(human programmed cell death 1); humanized mouse monoclonal [228-L-proline(H10-S>P)] γ 4 heavy chain (134-218')-disulfide with humanized mouse monoclonal κ light chain dimer (226-226":229-229")-bisdisulfide. The properties, uses, and preparation of pembrolizumab are described in International Patent Publication No. WO 2008/156712 A1, U.S. Patent No. 8,354,509 and U.S. Patent Application Publication Nos. US 2010/0266617 A1, US 2013/0108651 A1, and US 2013/0109843 A2, the disclosures of which are incorporated herein by reference. The clinical safety and efficacy of pembrolizumab in various forms of cancer is described in Fuerst, *Oncology Times*, **2014**, 36:35-36; Robert, *et al.*, *Lancet*, **2014**, 384:1109-17; and Thomas, *et al.*, *Exp. Opin. Biol. Ther.*, **2014**, 14:1061-1064. The amino acid sequences of pembrolizumab are set forth in Table 21. Pembrolizumab includes

the following disulfide bridges: 22-96, 22"-96", 23'-92', 23'''-92''', 134-218', 134"-218'', 138'-198', 138'''-198''', 147-203, 147"-203'', 226-226", 229-229", 261-321, 261"-321'', 367-425, and 367"-425'', and the following glycosylation sites (N): Asn-297 and Asn-297". Pembrolizumab is an IgG4/kappa isotype with a stabilizing S228P mutation in the Fc region; insertion of this mutation in the IgG4 hinge region prevents the formation of half molecules typically observed for IgG4 antibodies. Pembrolizumab is heterogeneously glycosylated at Asn297 within the Fc domain of each heavy chain, yielding a molecular weight of approximately 149 kDa for the intact antibody. The dominant glycoform of pembrolizumab is the fucosylated agalactodiantennary glycan form (G0F).

[00713] In an embodiment, a PD-1 inhibitor comprises a heavy chain given by SEQ ID NO:137 and a light chain given by SEQ ID NO:138. In an embodiment, a PD-1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:137 and SEQ ID NO:138, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:137 and SEQ ID NO:138, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:137 and SEQ ID NO:138, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:137 and SEQ ID NO:138, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:137 and SEQ ID NO:138, respectively. In an embodiment, a PD-1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:137 and SEQ ID NO:138, respectively. Representative sequences are listed in Table 17.

[00714] In an embodiment, the PD-1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of pembrolizumab. In an embodiment, the PD-1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:139, and the PD-1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:140, and conservative amino acid substitutions thereof. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:139 and SEQ ID NO:140, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L

regions that are each at least 98% identical to the sequences shown in SEQ ID NO:139 and SEQ ID NO:140, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:139 and SEQ ID NO:140, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:139 and SEQ ID NO:140, respectively. In an embodiment, a PD-1 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:139 and SEQ ID NO:140, respectively.

[00715] In an embodiment, a PD-1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:141, SEQ ID NO:142, and SEQ ID NO:143, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:144, SEQ ID NO:145, and SEQ ID NO:146, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-1 as any of the aforementioned antibodies.

[00716] In an embodiment, the PD-1 inhibitor is an anti-PD-1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to pembrolizumab. In an embodiment, the biosimilar comprises an anti-PD-1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is pembrolizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-1 antibody authorized or submitted for authorization, wherein the anti-PD-1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is pembrolizumab. The anti-PD-1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients

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

TABLE 17. Amino acid sequences for PD-1 inhibitors related to pembrolizumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:137 pembrolizumab heavy chain	QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 60 NEKFKNRVTL TTDSSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTITVTVSS 120 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 180 GLYSLSVVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPPCP APEFLGGPSV 240 FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY 300 RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK 360 NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTPPVLD S DGSFFLYSRL TVDKSRWQEG 420 NVFSCSVME ALHNHYTQKS LSLSLGK 447
SEQ ID NO:138 pembrolizumab light chain	EIVLTQSPAT LSLSFGERAT LSCRASKGVS TSGYSYLHWY QKPGQAPRL LIYLAAYLES 60 GVPARFSGSG SGTDFTLTIS SLEPEFAVY YCQHSRDLPL TFGGGTKVEI KRTVAAPSVF 120 IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSL 180 STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC 218
SEQ ID NO:139 pembrolizumab variable heavy chain	QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 60 NEKFKNRVTL TTDSSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTITVTVSS 120
SEQ ID NO:140 pembrolizumab variable light chain	EIVLTQSPAT LSLSFGERAT LSCRASKGVS TSGYSYLHWY QKPGQAPRL LIYLAAYLES 60 GVPARFSGSG SGTDFTLTIS SLEPEFAVY YCQHSRDLPL TFGGGTKVEI K 111
SEQ ID NO:141 pembrolizumab heavy chain CDR1	NYYMY 5
SEQ ID NO:142 pembrolizumab heavy chain CDR2	GINPSNGGTN FNEKFK 16
SEQ ID NO:143 pembrolizumab heavy chain CDR3	RDYRFDMGFD Y 11
SEQ ID NO:144 pembrolizumab light chain CDR1	RASKGVSTSG YSYLH 15
SEQ ID NO:145 pembrolizumab light chain CDR2	LASYLES 7
SEQ ID NO:146 pembrolizumab light chain CDR3	QHSRDLPLT 9

[00717] In an embodiment, the PD-1 inhibitor is a commercially-available anti-PD-1

monoclonal antibody, such as anti-m-PD-1 clones J43 (Cat # BE0033-2) and RMP1-14 (Cat # BE0146) (Bio X Cell, Inc., West Lebanon, NH, USA). A number of commercially-available anti-PD-1 antibodies are known to one of ordinary skill in the art.

[00718] In an embodiment, the PD-1 inhibitor is an antibody disclosed in U.S. Patent No. 8,354,509 or U.S. Patent Application Publication Nos. 2010/0266617 A1, 2013/0108651 A1, 2013/0109843 A2, the disclosures of which are incorporated by reference herein. In an embodiment, the PD-1 inhibitor is an anti-PD-1 antibody described in U.S. Patent Nos. 8,287,856, 8,580,247, and 8,168,757 and U.S. Patent Application Publication Nos. 2009/0028857 A1, 2010/0285013 A1, 2013/0022600 A1, and 2011/0008369 A1, the teachings of which are hereby incorporated by reference. In another embodiment, the PD-1 inhibitor is an anti-PD-1 antibody disclosed in U.S. Patent No. 8,735,553 B1, the disclosure of which is incorporated herein by reference. In an embodiment, the PD-1 inhibitor is pidilizumab, also known as CT-011, which is described in U.S. Patent No. 8,686,119, the disclosure of which is incorporated by reference herein.

[00719] In an embodiment, the PD-1 inhibitor may be a small molecule or a peptide, or a peptide derivative, such as those described in U.S. Patent Nos. 8,907,053; 9,096,642; and 9,044,442 and U.S. Patent Application Publication No. US 2015/0087581; 1,2,4-oxadiazole compounds and derivatives such as those described in U.S. Patent Application Publication No. 2015/0073024; cyclic peptidomimetic compounds and derivatives such as those described in U.S. Patent Application Publication No. US 2015/0073042; cyclic compounds and derivatives such as those described in U.S. Patent Application Publication No. US 2015/0125491; 1,3,4-oxadiazole and 1,3,4-thiadiazole compounds and derivatives such as those described in International Patent Application Publication No. WO 2015/033301; peptide-based compounds and derivatives such as those described in International Patent Application Publication Nos. WO 2015/036927 and WO 2015/04490, or a macrocyclic peptide-based compounds and derivatives such as those described in U.S. Patent Application Publication No. US 2014/0294898; the disclosures of each of which are hereby incorporated by reference in their entireties.

[00720] In an embodiment, the PD-L1 or PD-L2 inhibitor may be any PD-L1 or PD-L2 inhibitor, antagonist, or blocker known in the art. In particular, it is one of the PD-L1 or PD-L2 inhibitors, antagonist, or blockers described in more detail in the following paragraphs. The

terms “inhibitor,” “antagonist,” and “blocker” are used interchangeably herein in reference to PD-L1 and PD-L2 inhibitors. For avoidance of doubt, references herein to a PD-L1 or PD-L2 inhibitor that is an antibody may refer to a compound or antigen-binding fragments, variants, conjugates, or biosimilars thereof. For avoidance of doubt, references herein to a PD-L1 or PD-L2 inhibitor may refer to a compound or a pharmaceutically acceptable salt, ester, solvate, hydrate, cocrystal, or prodrug thereof.

[00721] In some embodiments, the compositions, processes and methods described herein include a PD-L1 or PD-L2 inhibitor. In some embodiments, the PD-L1 or PD-L2 inhibitor is a small molecule. In a preferred embodiment, the PD-L1 or PD-L2 inhibitor is an antibody (*i.e.*, an anti-PD-1 antibody), a fragment thereof, including Fab fragments, or a single-chain variable fragment (scFv) thereof. In some embodiments the PD-L1 or PD-L2 inhibitor is a polyclonal antibody. In a preferred embodiment, the PD-L1 or PD-L2 inhibitor is a monoclonal antibody. In some embodiments, the PD-L1 or PD-L2 inhibitor competes for binding with PD-L1 or PD-L2, and/or binds to an epitope on PD-L1 or PD-L2. In an embodiment, the antibody competes for binding with PD-L1 or PD-L2, and/or binds to an epitope on PD-L1 or PD-L2.

[00722] In some embodiments, the PD-L1 inhibitors provided herein are selective for PD-L1, in that the compounds bind or interact with PD-L1 at substantially lower concentrations than they bind or interact with other receptors, including the PD-L2 receptor. In certain embodiments, the compounds bind to the PD-L1 receptor at a binding constant that is at least about a 2-fold higher concentration, about a 3-fold higher concentration, about a 5-fold higher concentration, about a 10-fold higher concentration, about a 20-fold higher concentration, about a 30-fold higher concentration, about a 50-fold higher concentration, about a 100-fold higher concentration, about a 200-fold higher concentration, about a 300-fold higher concentration, or about a 500-fold higher concentration than to the PD-L2 receptor.

[00723] In some embodiments, the PD-L2 inhibitors provided herein are selective for PD-L2, in that the compounds bind or interact with PD-L2 at substantially lower concentrations than they bind or interact with other receptors, including the PD-L1 receptor. In certain embodiments, the compounds bind to the PD-L2 receptor at a binding constant that is at least about a 2-fold higher concentration, about a 3-fold higher concentration, about a 5-fold higher concentration, about a 10-fold higher concentration, about a 20-fold higher concentration, about a 30-fold higher

concentration, about a 50-fold higher concentration, about a 100-fold higher concentration, about a 200-fold higher concentration, about a 300-fold higher concentration, or about a 500-fold higher concentration than to the PD-L1 receptor.

[00724] Without being bound by any theory, it is believed that tumor cells express PD-L1, and that T cells express PD-1. However, PD-L1 expression by tumor cells is not required for efficacy of PD-1 or PD-L1 inhibitors or blockers. In an embodiment, the tumor cells express PD-L1. In another embodiment, the tumor cells do not express PD-L1. In some embodiments, the methods and compositions described herein include a combination of a PD-1 and a PD-L1 antibody, such as those described herein, in combination with a TIL. The administration of a combination of a PD-1 and a PD-L1 antibody and a TIL may be simultaneous or sequential.

[00725] In some embodiments, the compositions and methods described include a PD-L1 and/or PD-L2 inhibitor that binds human PD-L1 and/or PD-L2 with a K_D of about 100 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 90 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 80 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 70 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 60 pM or lower, a K_D of about 50 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 40 pM or lower, or binds human PD-L1 and/or PD-L2 with a K_D of about 30 pM or lower,

[00726] In some embodiments, the compositions and methods described include a PD-L1 and/or PD-L2 inhibitor that binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 9.5×10^5 1/M·s and/or faster, or binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 1×10^6 1/M·s or faster.

[00727] In some embodiments, the compositions and methods described include a PD-L1 and/or PD-L2 inhibitor that binds to human PD-L1 or PD-L2 with a k_{dissoc} of about 2×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human PD-1 with a k_{dissoc} of

about 2.6×10^{-5} 1/s or slower, binds to human PD-L1 or PD-L2 with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, or binds to human PD-L1 or PD-L2 with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[00728] In some embodiments, the compositions and methods described include a PD-L1 and/or PD-L2 inhibitor that blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 10 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 9 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 8 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 7 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 6 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 5 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 4 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 3 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 2 nM or lower; or blocks human PD-1, or blocks binding of human PD-L1 or human PD-L2 to human PD-1 with an IC_{50} of about 1 nM or lower.

[00729] In an embodiment, the PD-L1 inhibitor is durvalumab, also known as MEDI4736 (which is commercially available from Medimmune, LLC, Gaithersburg, Maryland, a subsidiary of AstraZeneca plc.), or antigen-binding fragments, conjugates, or variants thereof. In an embodiment, the PD-L1 inhibitor is an antibody disclosed in U.S. Patent No. 8,779,108 or U.S. Patent Application Publication No. 2013/0034559, the disclosures of which are incorporated by reference herein. The clinical efficacy of durvalumab has been described in Page, *et al.*, *Ann. Rev. Med.*, **2014**, 65:185-202; Brahmer, *et al.*, *J. Clin. Oncol.* **2014**, 32:5s (supplement, abstract 8021); and McDermott, *et al.*, *Cancer Treatment Rev.*, **2014**, 40:1056-64. The preparation and properties of durvalumab are described in U.S. Patent No. 8,779,108, the disclosure of which is incorporated by reference herein. The amino acid sequences of durvalumab are set forth in Table 18. The durvalumab monoclonal antibody includes disulfide linkages at 22-96, 22"-96", 23'-89', 23'''-89''', 135'-195', 135'''-195''', 148-204, 148"-204", 215'-224, 215'''-224", 230-230", 233-233", 265-325, 265"-325", 371-429, and 371"-429"; and N-glycosylation sites at Asn-301 and Asn-301".

[00730] In an embodiment, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:147 and a light chain given by SEQ ID NO:148. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:147 and SEQ ID NO:148, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:147 and SEQ ID NO:148, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:147 and SEQ ID NO:148, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:147 and SEQ ID NO:148, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:147 and SEQ ID NO:148, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:147 and SEQ ID NO:148, respectively.

[00731] In an embodiment, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of durvalumab. In an embodiment, the PD-L1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:149, and the PD-L1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:150, and conservative amino acid substitutions thereof. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:149 and SEQ ID NO:150, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:149 and SEQ ID NO:150, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:149 and SEQ ID NO:150, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:149 and SEQ ID NO:150, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:149 and SEQ ID NO:150, respectively.

[00732] In an embodiment, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3

domains having the sequences set forth in SEQ ID NO:151, SEQ ID NO:152, and SEQ ID NO:153, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:154, SEQ ID NO:155, and SEQ ID NO:156, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

[00733] In an embodiment, the PD-L1 inhibitor is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to durvalumab. In an embodiment, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is durvalumab.

TABLE 18. Amino acid sequences for PD-L1 inhibitors related to durvalumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)						
SEQ ID NO:147 durvalumab heavy chain	EVQLVESGGG	LVQPGGSLRL	SCAASGFTFS	RYWMSWVRQA	PGKGLEWVAN	IKQDGSEKYY	60
	VDSVKGRFTI	SRDNAKNSLY	LQMNSLRAED	TAVYYCAREG	GWFGELAFDY	WGQGTILVTVS	120
	SASTKGPSVF	PLAPSSKSTS	GGTAALGCLV	KDYFPEPVTV	SWNSGALTSG	VHTFPAVLQS	180
	SGLYSLSSVV	TVPSSSLGTQ	TYICNVNHKP	SNTKVDKRVK	PKSCDKHTC	PPCPAPEFEG	240
	GPSVFLFPPK	PKDTLMISRT	PEVTCVVDV	SHEDPEVKFN	WYVDGVEVHN	AKTKPREEQY	300
	NSTYRVVSVL	TVLHQDWLNG	KEYKCKVSNK	ALPASIEKTI	SKAKGQPREP	QVYTLPPSRE	360
	EMTKNQVSLT	CLVKGFYPSD	IAVEWESNGQ	PENNYKTTTP	VLDSDGSFFL	YSKLTVDKSR	420
	WQQGNVFSCS	VMHEALHNNH	TQKSLSLSPG	K			451
SEQ ID NO:148 durvalumab light chain	EVQLVESGGG	LVQPGGSLRL	SCAASGFTFS	RYWMSWVRQA	PGKGLEWVAN	EIVLTQSPGT	60
	LSLSPGERAT	LSCRASQKVS	SSYLAWYQQK	PGQAPRLLIY	DASSRATGIP	DRFSGSGSGT	120
	DFTLTISRLE	PEDEAVYYCQ	QYGSLLPWTFG	QGTKVEIKRT	VAAPSVFIFP	PSDEQLKSGT	180
	ASVVCLLNMF	YPREAKVQWK	VDNALQSGNS	QESVTEQDSK	DSTYSLSTL	TLSKADYEKH	240
	KVYACEVTHQ	GLSSPVTKSF	NRGEC				265
SEQ ID NO:149 durvalumab variable heavy chain	EVQLVESGGG	LVQPGGSLRL	SCAASGFTFS	RYWMSWVRQA	PGKGLEWVAN	IKQDGSEKYY	60
	VDSVKGRFTI	SRDNAKNSLY	LQMNSLRAED	TAVYYCAREG	GWFGELAFDY	WGQGTILVTVS	120
	S						121
SEQ ID NO:150 durvalumab variable light chain	EIVLTQSPGT	LSLSPGERAT	LSCRASQKVS	SSYLAWYQQK	PGQAPRLLIY	DASSRATGIP	60
	DRFSGSGSGT	DFTLTISRLE	PEDEAVYYCQ	QYGSLLPWTFG	QGTKVEIK		108
SEQ ID NO:151 durvalumab heavy chain CDR1	RYWMS						5
SEQ ID NO:152 durvalumab heavy chain CDR2	NIKQDGSEKY YVDSVKG						17
SEQ ID NO:153 durvalumab heavy chain CDR3	EGGWFGELAF DY						12
SEQ ID NO:154 durvalumab light chain CDR1	RASQKRVSSSY LA						12
SEQ ID NO:155 durvalumab light chain CDR2	DASSRAT						7
SEQ ID NO:156 durvalumab light chain CDR3	QQYGSLLPWT						9

[00734] In an embodiment, the PD-L1 inhibitor is avelumab, also known as MSB0010718C (commercially available from Merck KGaA/EMD Serono), or antigen-binding fragments, conjugates, or variants thereof. The preparation and properties of avelumab are described in U.S. Patent Application Publication No. US 2014/0341917 A1, the disclosure of which is specifically incorporated by reference herein. The amino acid sequences of avelumab are set forth in Table 19. Avelumab has intra-heavy chain disulfide linkages (C23-C104) at 22-96, 147-203, 264-324, 370-428, 22"-96", 147"-203", 264"-324", and 370"-428"; intra-light chain disulfide linkages (C23-C104) at 22'-90', 138'-197', 22'''-90''', and 138'''-197'''; intra-heavy-light chain disulfide linkages (h 5-CL 126) at 223-215' and 223"-215''"; intra-heavy-heavy chain disulfide linkages (h

11, h 14) at 229-229" and 232-232"; N-glycosylation sites (H CH₂ N84.4) at 300, 300"; fucosylated complex bi-antennary CHO-type glycans; and H CHS K2 C-terminal lysine clipping at 450 and 450'.

[00735] In an embodiment, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:157 and a light chain given by SEQ ID NO:158. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:157 and SEQ ID NO:158, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:157 and SEQ ID NO:158, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:157 and SEQ ID NO:158, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:157 and SEQ ID NO:158, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:157 and SEQ ID NO:158, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:157 and SEQ ID NO:158, respectively.

[00736] In an embodiment, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of avelumab. In an embodiment, the PD-L1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:159, and the PD-L1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:160, and conservative amino acid substitutions thereof. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:159 and SEQ ID NO:160, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:160 and SEQ ID NO:160, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:159 and SEQ ID NO:160, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:159 and SEQ ID NO:160, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that

are each at least 95% identical to the sequences shown in SEQ ID NO:159 and SEQ ID NO:160, respectively.

[00737] In an embodiment, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:161, SEQ ID NO:162, and SEQ ID NO:163, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:164, SEQ ID NO:165, and SEQ ID NO:166, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

[00738] In an embodiment, the PD-L1 inhibitor is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to avelumab. In an embodiment, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab.

product is avelumab.

TABLE 19. Amino acid sequences for PD-L1 inhibitors related to avelumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:157 avelumab heavy chain	EVQLLES GGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS IYPSGGITFY 60 ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK LGTVTTVDYW GQGTLLTVSS 120 ASTKGPSVFAP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 180 GLYSLSSVVT VPSSSLGTQT YICNVNKKPS NTKVDKKVEP KSCDKTHTCP PCPAPPELLGG 240 PSVFLFPPKP KDTLMISRTPEVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN 300 STYRVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE 360 LTKNQVSLTCLVKGFYPSDI AVEWESNGQP ENNYKTTTPV LDSDGSFFLY SKLTVDKSRW 420 QQGNVFSCSV MHEALHNHYT QKSLSLSPGK 450
SEQ ID NO:158 avelumab light chain	QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLM IYDVSNRPSGV 60 SNRFGSGSKG NTASLTISGL QAEDEADYYC SSYTSSSTRV FGTGKVTVL GQPKANPTVT 120 LFPPSSEELQ ANKATLVCLI SDFYPGAVTV AWKADGSPVK AGVETTKPSK QSNNKYAASS 180 YLSLTPEQWK SHRSYSCQVT HEGSTVEKTV APTES 216
SEQ ID NO:159 avelumab variable heavy chain	EVQLLES GGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS IYPSGGITFY 60 ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK LGTVTTVDYW GQGTLLTVSS 120
SEQ ID NO:160 avelumab variable light chain	QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLM IYDVSNRPSGV 60 SNRFGSGSKG NTASLTISGL QAEDEADYYC SSYTSSSTRV FGTGKVTVL 110
SEQ ID NO:161 avelumab heavy chain CDR1	SYIMM 5
SEQ ID NO:162 avelumab heavy chain CDR2	SIYPSGGITF YADTVKG 17
SEQ ID NO:163 avelumab heavy chain CDR3	IKLGTVTTVD Y 11
SEQ ID NO:164 avelumab light chain CDR1	TGTSSDVGGY NYVS 14
SEQ ID NO:165 avelumab light chain CDR2	DVSNRPS 7
SEQ ID NO:166 avelumab light chain CDR3	SSYTSSSTRV 10

[00739] In an embodiment, the PD-L1 inhibitor is atezolizumab, also known as MPDL3280A or RG7446 (commercially available as TECENTRIQ from Genentech, Inc., a subsidiary of Roche Holding AG, Basel, Switzerland), or antigen-binding fragments, conjugates, or variants thereof. In an embodiment, the PD-L1 inhibitor is an antibody disclosed in U.S. Patent No. 8,217,149, the disclosure of which is specifically incorporated by reference herein. In an embodiment, the PD-L1 inhibitor is an antibody disclosed in U.S. Patent Application Publication Nos. 2010/0203056 A1, 2013/0045200 A1, 2013/0045201 A1, 2013/0045202 A1, or 2014/0065135 A1, the

disclosures of which are specifically incorporated by reference herein. The preparation and properties of atezolizumab are described in U.S. Patent No. 8,217,149, the disclosure of which is incorporated by reference herein. The amino acid sequences of atezolizumab are set forth in Table 20. Atezolizumab has intra-heavy chain disulfide linkages (C23-C104) at 22-96, 145-201, 262-322, 368-426, 22"-96", 145"-201", 262"-322", and 368"-426"; intra-light chain disulfide linkages (C23-C104) at 23'-88', 134'-194', 23'''-88'''', and 134'''-194''''; intra-heavy-light chain disulfide linkages (h 5-CL 126) at 221-214' and 221"-214''; intra-heavy-heavy chain disulfide linkages (h 11, h 14) at 227-227" and 230-230"; and N-glycosylation sites (H CH₂ N84.4>A) at 298 and 298'.

[00740] In an embodiment, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:167 and a light chain given by SEQ ID NO:168. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:167 and SEQ ID NO:168, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:167 and SEQ ID NO:168, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:167 and SEQ ID NO:168, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:167 and SEQ ID NO:168, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:167 and SEQ ID NO:168, respectively. In an embodiment, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:167 and SEQ ID NO:168, respectively.

[00741] In an embodiment, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of atezolizumab. In an embodiment, the PD-L1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:169, and the PD-L1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:170, and conservative amino acid substitutions thereof. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:169 and SEQ ID NO:170, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H

and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:169 and SEQ ID NO:170, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:169 and SEQ ID NO:170, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:169 and SEQ ID NO:170, respectively. In an embodiment, a PD-L1 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:169 and SEQ ID NO:170, respectively.

[00742] In an embodiment, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:171, SEQ ID NO:172, and SEQ ID NO:173, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:174, SEQ ID NO:175, and SEQ ID NO:176, respectively, and conservative amino acid substitutions thereof. In an embodiment, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

[00743] In an embodiment, the anti-PD-L1 antibody is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to atezolizumab. In an embodiment, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is atezolizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is atezolizumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or

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

TABLE 20. Amino acid sequences for PD-L1 inhibitors related to atezolizumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:167 atezolizumab heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS DSWIHWRQA PGKGLEWVAW ISPYGGSTYY 60 ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCARRH WPGGFDYWGQ GTLTVSSAS 120 TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL 180 YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTCPPC PAPELLGGPS 240 VFLFPPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGEVEVHNAKT KPREEQYAST 300 YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT 360 KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ 420 GNVFSCSVMH EALHNHYTQK SLSLSPGK 448
SEQ ID NO:168 atezolizumab light chain	DIQMTQSPSS LSASVGDRVT ITCRASQDVS TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS 60 RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YLYHPATFGQ GTKVEIKRTV AAPSVFIAPP 120 SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSLTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEK 214
SEQ ID NO:169 atezolizumab variable heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS DSWIHWRQA PGKGLEWVAW ISPYGGSTYY 60 ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCARRH WPGGFDYWGQ GTLTVSA 118
SEQ ID NO:170 atezolizumab variable light chain	DIQMTQSPSS LSASVGDRVT ITCRASQDVS TAVAWYQQKP GKAPKLLIYS ASFLYSGVPS 60 RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YLYHPATFGQ GTKVEIKR 108
SEQ ID NO:171 atezolizumab heavy chain CDR1	GFTFSDSWIH 10
SEQ ID NO:172 atezolizumab heavy chain CDR2	AWISPYGGST YYADSVKG 18
SEQ ID NO:173 atezolizumab heavy chain CDR3	RHWPGGFDY 9
SEQ ID NO:174 atezolizumab light chain CDR1	RASQDVSTAV A 11
SEQ ID NO:175 atezolizumab light chain CDR2	SASFLYS 7
SEQ ID NO:176 atezolizumab light chain CDR3	QQYLYHPAT 9

[00744] While preferred embodiments of the invention are shown and described herein, such embodiments are provided by way of example only and are not intended to otherwise limit the scope of the invention. Various alternatives to the described embodiments of the invention may be employed in practicing the invention. Therefore, the spirit and scope of the appended claims should not be limited to the description of the preferred versions contained herein.

[00745] The reader's attention is directed to all papers and documents which are filed concurrently with this specification and which are open to public inspection with this specification, and the contents of all such papers and documents incorporated herein by reference. All the features disclosed in the specification (including any accompanying claims, abstract, and drawings) may be replaced by alternative features serving the same, equivalent or similar purpose, unless expressly stated otherwise. Thus, unless expressly stated otherwise, each feature disclosed is one example only of a generic series of equivalent or similar features.

EXAMPLES

[00746] The embodiments encompassed herein are now described with reference to the following examples. These examples are provided for the purpose of illustration only and the disclosure encompassed herein should in no way be construed as being limited to these examples, but rather should be construed to encompass any and all variations which become evident as a result of the teachings provided herein.

Example 1. Preparation of Media for Pre-REP and REP Processes.

[00747] This Example describes the procedure for the preparation of tissue culture media for use in protocols involving the culture of tumor infiltrating lymphocytes (TIL) derived from various tumor types including, but not limited to, metastatic melanoma, head and neck squamous cell carcinoma (HNSCC), ovarian carcinoma, triple-negative breast carcinoma, and lung adenocarcinoma. This media can be used for preparation of any of the TILs described in the present application and Examples.

Preparation of CM1

[00748] Removed the following reagents from cold storage and warmed them in a 37°C water bath: (RPMI1640, Human AB serum, 200 mM L-glutamine). Prepared CM1 medium according

to Table 21 below by adding each of the ingredients into the top section of a 0.2 μ m filter unit appropriate to the volume to be filtered. Store at 4°C.

TABLE 21. Preparation of CM1

Ingredient	Final Concentration	Final Volume 500 ml	Final Volume 1L
RPMI1640	NA	450 mL	900 mL
Human AB serum, heat-inactivated 10%	50 mL	100 mL	
200mM L-glutamine	2 mM	5 mL	10 mL
55mM BME	55 μ M	0.5 mL	1 mL
50mg/ml gentamicin sulfate	50 μ g/ml	0.5 mL	1 mL

[00749] On the day of use, prewarmed required amount of CM1 in 37°C water bath and add 6000 IU/mL IL-2.

[00750] Additional supplementation as needed according to Table 22.

TABLE 22. Additional Supplementation of CM1, as needed.

Supplement	Stock concentration	Dilution	Final concentration
GlutaMAX™	200 mM	1:100	2 mM
Penicillin/streptomycin	10,000 U/mL penicillin 10,000 μ g/mL streptomycin	1:100	100 U/mL penicillin 100 μ g/mL streptomycin
Amphotericin B	250 μ g/mL	1:100	2.5 μ g/mL

Preparation of CM2

[00751] Removed prepared CM1 from refrigerator or prepare fresh CM1 as above. Removed AIM-V® from refrigerator and prepared the amount of CM2 needed by mixing prepared CM1 with an equal volume of AIM-V® in a sterile media bottle. Added 3000 IU/mL IL-2 to CM2 medium on the day of usage. Made sufficient amount of CM2 with 3000 IU/mL IL-2 on the day

of usage. Labeled the CM2 media bottle with its name, the initials of the preparer, the date it was filtered/prepared, the two-week expiration date and store at 4°C until needed for tissue culture.

Preparation of CM3

[00752] Prepared CM3 on the day it was required for use. CM3 was the same as AIM-V® medium, supplemented with 3000 IU/mL IL-2 on the day of use. Prepared an amount of CM3 sufficient to experimental needs by adding IL-2 stock solution directly to the bottle or bag of AIM-V. Mixed well by gentle shaking. Label bottle with “3000 IU/mL IL-2” immediately after adding to the AIM-V. If there was excess CM3, stored it in bottles at 4°C labeled with the media name, the initials of the preparer, the date the media was prepared, and its expiration date (7 days after preparation). Discarded media supplemented with IL-2 after 7 days of storage at 4°C.

Preparation of CM4

[00753] CM4 was the same as CM3, with the additional supplement of 2 mM GlutaMAX™ (final concentration). For every 1L of CM3, added 10mL of 200 mM GlutaMAX™. Prepared an amount of CM4 sufficient to experimental needs by adding IL-2 stock solution and GlutaMAX™ stock solution directly to the bottle or bag of AIM-V. Mixed well by gentle shaking. Labeled bottle with “3000 IU/mL IL-2 and GlutaMAX” immediately after adding to the AIM-V. If there was excess CM4, stored it in bottles at 4°C labeled with the media name, “GlutaMAX”, and its expiration date (7 days after preparation). Discarded media supplemented with IL-2 after 7-days of storage at 4°C.

Example 2. Use of IL-2, IL-15, and IL-21 Cytokine Cocktail.

[00754] This example describes the use of IL-2, IL-15, and IL-21 cytokines, which serve as additional T cell growth factors, in combination with the TIL process of any of the examples or embodiments herein, including examples and embodiments that encompass cryopreservation of tumor tissue or tumor fragments.

[00755] Using the processes described herein, TILs were grown from colorectal, melanoma, cervical, triple negative breast, lung and renal tumors in presence of IL-2 in one arm of the experiment and, in place of IL-2, a combination of IL-2, IL-15, and IL-21 in another arm at the initiation of culture. At the completion of the pre-REP, cultures were assessed for expansion, phenotype, function (CD107a⁺ and IFN-γ) and TCR Vβ repertoire. IL-15 and IL-21 are described

elsewhere herein and in Gruijl, *et al.*, IL-21 promotes the expansion of CD27⁺CD28⁺ tumor infiltrating lymphocytes with high cytotoxic potential and low collateral expansion of regulatory T cells, Santegoets, S. J., *J. Transl Med.*, 11:37 (2013) available at <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3626797/>.

[00756] The results showed that enhanced TIL expansion (>20%), in both CD4⁺ and CD8⁺ cells in the IL-2, IL-15, and IL-21 treated conditions were observed in multiple histologies relative to the IL-2 only conditions. There was a skewing towards a predominantly CD8⁺ population with a skewed TCR V β repertoire in the TILs obtained from the IL-2, IL-15, and IL-21 treated cultures relative to the IL-2 only cultures. IFN- γ and CD107A were elevated in the IL-2, IL-15, and IL-21 treated TILs, in comparison to TILs treated only IL-2.

Example 3. Preparation of IL-2 Stock Solution.

[00757] This Example describes the process of dissolving purified, lyophilized recombinant human interleukin-2 into stock samples suitable for use in further tissue culture protocols, including all of those described in the present application and Examples, including those that involve using rhIL-2.

Procedure

[00758] Prepared 0.2% Acetic Acid solution (HAc). Transferred 29 mL sterile water to a 50 mL conical tube. Added 1 mL 1N (1 normal) acetic acid to the 50 mL conical tube. Mixed well by inverting tube 2-3 times. Sterilized the HAc solution by filtration using a Steriflip filter

[00759] Prepare 1% HSA in PBS. Added 4 mL of 25% HSA stock solution to 96 mL PBS in a 150 mL sterile filter unit. Filtered solution. Stored at 4°C. For each vial of rhIL-2 prepared, fill out forms.

[00760] Prepared rhIL-2 stock solution (6×10^6 IU/mL final concentration). Each lot of rhIL-2 was different and required information found in the manufacturer's Certificate of Analysis (COA), such as: 1) Mass of rhIL-2 per vial (mg), 2) Specific activity of rhIL-2 (IU/mg) and 3) Recommended 0.2% HAc reconstitution volume (mL).

[00761] Calculated the volume of 1% HSA required for rhIL-2 lot by using the equation below:

$$\left(\frac{\text{Vial Mass (mg)} \times \text{Biological Activity } \left(\frac{\text{IU}}{\text{mg}} \right)}{6 \times 10^6 \frac{\text{IU}}{\text{mL}}} \right) - \text{HAc vol (mL)} = 1\% \text{ HSA vol (mL)}$$

[00762] For example, according to CellGenix's rhIL-2 lot 10200121 COA, the specific activity for the 1 mg vial is 25×10^6 IU/mg. It recommends reconstituting the rhIL-2 in 2 mL 0.2% HAc.

$$\left(\frac{1 \text{ mg} \times 25 \times 10^6 \frac{\text{IU}}{\text{mg}}}{6 \times 10^6 \frac{\text{IU}}{\text{mL}}} \right) - 2 \text{ mL} = 2.167 \text{ mL HSA}$$

[00763] Wiped rubber stopper of IL-2 vial with alcohol wipe. Using a 16G needle attached to a 3 mL syringe, injected recommended volume of 0.2% HAc into vial. Took care to not dislodge the stopper as the needle is withdrawn. Inverted vial 3 times and swirled until all powder is dissolved. Carefully removed the stopper and set aside on an alcohol wipe. Added the calculated volume of 1% HSA to the vial.

[00764] Storage of rhIL-2 solution. For short-term storage (<72 hrs), stored vial at 4°C. For long-term storage (>72 hrs), aliquoted vial into smaller volumes and stored in cryovials at -20°C until ready to use. Avoided freeze/thaw cycles. Expired 6 months after date of preparation. Rh-IL-2 labels included vendor and catalog number, lot number, expiration date, operator initials, concentration and volume of aliquot.

Example 4. TIL Cryopreservation Processes.

[00765] This example describes the cryopreservation process method for TILs prepared with the abbreviated, closed procedure described in Example 5 using the CryoMed Controlled Rate Freezer, Model 7454 (Thermo Scientific).

[00766] The equipment used was as follows: aluminum cassette holder rack (compatible with CS750 freezer bags), cryostorage cassettes for 750 mL bags, low pressure (22 psi) liquid nitrogen tank, refrigerator, thermocouple sensor (ribbon type for bags), and CryoStore CS750 Freezing bags (OriGen Scientific).

[00767] The freezing process provides for a 0.5 °C rate from nucleation to -20 °C and 1 °C per minute cooling rate to -80 °C end temperature. The program parameters are as follows: Step 1 - wait at 4 °C; Step 2: 1.0 °C/min (sample temperature) to -4 °C; Step 3: 20.0 °C/min (chamber temperature) to -45 °C; Step 4: 10.0 °C/min (chamber temperature) to -10.0 °C; Step 5: 0.5 °C/min (chamber temperature) to -20 °C; and Step 6: 1.0 °C/min (sample temperature) to -80 °C.

Example 5. Production of a Cryopreserved TIL Cell Therapy Using a Closed System.

[00768] This example describes the procedure for cGMP manufacture of TIL cell therapy process in gas permeable containers, such as G-Rex Flasks (Wilson Wolf Manufacturing Corp., New Brighton, MN, USA), according to current Good Tissue Practices and current Good Manufacturing Practices. This material will be manufactured under US FDA Good Manufacturing Practices Regulations (21 CFR Part 210, 211, 1270, and 1271), and applicable ICH Q7 standards for Phase I through Commercial Material. This method may be combined with the tumor cryopreservation and thawing processes described herein.

[00769] The process summary is provided in Table 23 below.

TABLE 23. Process Summary.

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

[00770] Throughout this Example, it is assumed that 1.0 mL/L = 1.0 g/kg, unless otherwise specified. Once opened, the following expiries apply at 2°C – 8°C: Human Serum, type AB (HI) Gemini, 1 month; 2-mercaptoethanol, 1 month. Gentamicin Sulfate, 50 mg/mL stock may be kept at room temperature for 1 month. Bags containing 10L of AIM-V media may be warmed at

room temperature once only for up to 24 hours prior to use. During the Day 22 harvest two Gatherex™ pumps may be used to harvest the TIL from the G-Rex500MCS flasks.

Day 0 – CM1 Media Preparation

[00771] Prepare RPMI 1640 Media. In the BSC, using an appropriately sized pipette, remove 100.0 mL from 1000 mL RPMI 1640 Media and place into an appropriately sized container labeled “Waste.”

[00772] In the BSC, add reagents to RPMI 1640 Media bottle. Add the following reagents to the RPMI 1640 Media bottle as shown in Table 21. Record 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)

[00773] Cap RPMI 1640 Media bottle and swirl bottle to ensure reagents were mixed thoroughly. Filter RPMI 1640 Media through 1L 0.22-micron filter unit. Label filtered media. Aseptically cap the filtered media and label.

[00774] Thaw one 1.1 mL IL-2 aliquot (6×10^6 IU/mL) (BR71424) until all ice had melted. Transfer IL-2 stock solution to media. In the BSC, transfer 1.0 mL of IL-2 stock solution to the CM1 Day 0 Media Bottle prepared above. Add CM1 Day 0 Media 1 bottle and IL-2 (6×10^6 IU/mL) 1.0 mL. Cap and swirl the bottle to mix media containing IL-2. Relabeled as “Complete CM1 Day 0 Media”.

[00775] Remove 20.0 mL of media using an appropriately sized pipette and dispensed into a 50mL conical tube. In BSC, transfer 25.0 mL of “Complete CM1 Day 0 Media” to a 50 mL conical tube. Label the tube as “Tissue Pieces”. Aseptically pass G-Rex100MCS (W3013130) into the BSC. In the BSC, closed all clamps on the G-Rex100MCS, leaving vent filter clamp open. Connect the red line of G-Rex100MCS flask to the larger diameter end of the repeater pump fluid transfer set (W3009497) via luer connection. Stage Baxa pump next to BSC. Remove pump tubing section of repeater pump fluid transfer set from BSC and install in repeater pump. Within the BSC, remove the syringe from Pumpmatic Liquid-Dispensing System (PLDS) (W3012720) and discard.

[00776] Connect 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” for aspiration. Open

all clamps between media and G-Rex100MCS. Pump 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 is transferred, clear the line and stop pump.

[00777] Disconnect pump from flask. Ensure all clamps were closed on the flask, except vent filter. Remove the repeater pump fluid transfer set from the red media line, and place a red cap (W3012845) on the red media line. Remove G-Rex100MCS flask from BSC, heat seal off the red cap from the red line near the terminal luer. Incubator parameters: 37.0 ± 2.0 °C; CO₂ Percentage: 5.0 ± 1.5 %CO₂.

[00778] Place the 50mL conical tube” in incubator for ≥ 30 minutes of warming.

Day 0 – Tumor Wash Media Preparation

[00779] Add gentamicin to HBSS. In the BSC, add 5.0 mL gentamicin (W3009832 or W3012735) to 1 x 500 mL HBSS Media (W3013128) bottle. Record volumes. Add per bottle: HBSS (500.0 mL); Gentamicin sulfate, 50 mg/ml (5.0 mL). Mix reagents thoroughly. Filter HBSS containing gentamicin through a 1L 0.22-micron (0.22 μ m) filter unit (W1218810). Aseptically cap the filtered media and labeled with the following information.

Day 0 – Tumor Processing

[00780] Obtain tumor specimen (from cryopreserved tumor) and transfer into suite at 2°C – 8°C immediately for processing and recorded tumor information.

[00781] Wash tumor specimens three times at 2°C – 8°C, with gentle agitation for at least 3 minutes for each wash. The wash solution is removed and replaced with fresh solution after each wash.

[00782] Using a transfer pipette, place 4 individual drops of Tumor Wash Media from the conical into each of the 6 circles on the upturned lids of the 6-well plates (2 lids). Place an extra drop on two circles for a total of 50 drops.

[00783] Tumor Wash 3: Using forceps, remove the tumor from the “Wash 2” dish and transferred to the “Wash 3” dish. Using forceps, wash tumor specimen by gently agitating and allow it to sit for ≥ 3 minutes. Record time.

[00784] Place a ruler under 150 mm dish lid. Using forceps, aseptically transferred tumor specimen to the 150 mm dissection dish lid. Arrange all pieces of tumor specimen end to end and

recorded the approximate overall length and number of fragments. Assess the tumor for necrotic/fatty tissue. Assess whether > 30% of entire tumor area observed to be necrotic and/or fatty tissue; if yes, ensure tumor was of appropriate size if so proceed. Assess whether < 30% of entire tumor area were observed to be necrotic or fatty tissue; if yes, proceed.

[00785] 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. To maintain tumor internal structure, use only vertical cutting pressure. Did not cut in a sawing motion with scalpel.

[00786] Using a combination of scalpel and/or forceps, cut the tumor specimen into even, appropriately sized fragments (up to 6 intermediate fragments). To maintain tumor internal structure, use only vertical cutting pressure. Again, do not cut in a sawing motion with scalpel. Keep non-dissected intermediate fragments completely submerged in “Tumor Wash Media”. Transfer each intermediate fragment to the “holding” dish.

[00787] Manipulate one intermediate fragment at a time, dissected the tumor intermediate fragment in the dissection dish into pieces approximately $3 \times 3 \times 3$ mm in size, minimizing the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. To maintain tumor internal structure, use only vertical cutting pressure. Again, do not cut in a sawing motion with scalpel.

[00788] Select up to eight (8) tumor pieces without hemorrhagic, necrotic, and/or fatty tissue. Used the ruler for reference. Continue dissection until 8 favorable pieces have been obtained, or the entire intermediate fragment has been dissected. Transfer each selected piece to one of the drops of “Tumor Wash Media”.

[00789] After selecting up to eight (8) pieces from the intermediate fragment, place remnants of intermediate fragment into a new single well of “favorable intermediate fragments” 6-well plate.

[00790] 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.

[00791] Remove the “Tissue Pieces” 50 mL conical tube from the incubator. Ensured conical tube was warmed for ≥ 30 min. Pass “Tissue Pieces” 50 mL conical into the BSC, ensuring not to compromise the sterility of open processing surfaces.

[00792] Using a transfer pipette, scalpel, forceps or combination, transfer the selected 50 best tumor fragments from favorable dish lids to the “Tissue Pieces” 50 mL conical tube. 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.

[00793] Remove G-Rex100MCS containing media from incubator. Aseptically passed G-Rex100MCS flask into the BSC. When transferring the flask, do not hold from the lid or the bottom of the vessel. Transfer the vessel by handling the sides. In the BSC, lift G-Rex100MCS flask cap, ensuring that sterility of internal tubing was maintained. Swirl conical tube with tumor pieces to suspend and quickly pour the contents into the G-Rex100MCS flask. Ensure that the tumor pieces are evenly distributed across the membrane of the flask. Gently tilted the flask back and forth if necessary to evenly distribute the tumor pieces. Record 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, contacted Area Management, and documented in Section 10.0.

[00794] Incubate G-Rex100MCS at the following parameters: Incubate G-Rex flask: Temperature LED Display: 37.0 ± 2.0 °C; CO₂ Percentage: 5.0 ± 1.5 % CO₂. Perform 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.

Day 11 – Media Preparation

[00795] Monitor Incubator. Incubator parameters: Temperature LED Display: 37.0 ± 2.0 °C; CO₂ Percentage: 5.0 ± 1.5 %CO₂. Warm 3×1000 mL RPMI 1640 Media (W3013112) bottles and 3×1000 mL AIM-V (W3009501) bottles in an incubator for ≥ 30 minutes. Recorded time. Media: RPMI 1640 and AIM-V. Placed an additional 1×1000 mL bottle of AIM-V Media (W3009501) at room temperature for further use.

[00796] Remove the RPMI 1640 Media when time was reached. Record end incubation time in previous step. Ensure media is warmed for ≥ 30 min. In the BSC, removed 100.0 mL from each

of the three pre-warmed 1000 mL RPMI 1640 Media bottles and place into an appropriately sized container labeled “Waste”. In the BSC, add the following reagents to each of the three RPMI 1640 Media bottles and recorded volumes added to each bottle. GemCell Human serum, Heat Inactivated Type AB (100.0 mL), GlutaMax (10.0 mL), Gentamicin sulfate, 50 µg/mL (1.0 mL), 2-mercaptoethanol (1.0 mL).

[00797] Cap bottles and swirl to ensure reagents were mixed thoroughly. Filtered each bottle of media through a separate 1L 0.22-micron filter unit. Aseptically cap the filtered media and label each bottle with CM1 Day 11 Media. Thawed 3×1.1 mL aliquots of IL-2 (6×10^6 IU/mL) (BR71424) until all ice had melted Recorded IL 2 lot # and expiration date/time.

[00798] Remove the three bottles of AIM-V Media from the incubator. Record end incubation time. Ensure media had been warmed for ≥ 30 minutes. Using a micropipette, add 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. Record the total volume added. Label bottle as “AIM-V Containing IL-2”. Aseptically transfer a 10L Labtainer Bag and a repeater pump transfer set into the BSC. Closed all lines on a 10L Labtainer bag. Attach 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.

[00799] Stage the Baxa pump next to the BSC. Feed the transfer set tubing through the Baxa pump. Set the Baxa Pump to “High” and “9”. Remove syringe from Pumpmatic Liquid-Dispensing System (PLDS) and discarded. Do not compromise the sterility of the PLDS pipette.

[00800] Connect the PLDS pipette to smaller diameter end of repeater pump fluid transfer set via luer connection and place pipette tip in AIM-V media containing IL-2 bottle for aspiration. Opened all clamps between media bottle and 10L Labtainer.

[00801] Using the PLDS, transfer pre-warmed AIM-V media containing IL-2 prepared, as well as two additional AIM-V bottles into the 10L Labtainer bag. Add the three bottles of filtered CM1 Day 11 Media. After addition of final bottle, clear the line to the bag. Stop the pump between addition of each bottle of media. Remove PLDS from the transfer set and place a red cap on the luer of the line in the BSC. Gently massage the bag to mix. Label the media bag with the following information. Expiration date is 24 hours from the preparation date.

[00802] Attach a 60 mL syringe to the available female port of the “Complete CM2 Day 11 Media” bag. Remove 20.0 mL of media and place in a 50 mL conical tube. Place a red cap on the female port of the “Complete CM2 Day 11 Media” Bag. Label and stored Media Retain Sample at 2°C-8°C until submitted for testing. Heat seal off the red cap on the transfer set line, close to red cap. Keep the transfer set on the bag.

[00803] In the BSC, add 4.5 mL of AIM-V Media that had 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 “Feeder” and vial number (1-4). Transfer any remaining 2-mercaptoethanol, GlutaMax, and human serum from the BSC to 2°C to 8°C.

[00804] Outside of the BSC, weld a 1L Transfer Pack to the transfer set attached to the “Complete CM2 Day 11 Media” bag prepared. Labeled transfer pack as “Feeder Cell CM2 Media” and lot number. Make a mark on the tubing of the 1L Transfer Pack tubing a few inches away from the bag. Place the empty Transfer Pack onto the scale so that the tubing was on the scale to the point of the mark. Tare the scale and leave the empty Transfer Pack on the scale.

[00805] Set the Baxa pump to “Medium” and “4.” Pump 500.0 ± 5.0 mL of “Complete CM2 Day 11” media into Cell CM2 Media” transfer pack. Measure by weight and record the volume of Complete CM2 media added to the Transfer Pack.

[00806] Once filled, heat seal the line. Separate CM2 Day 11 media bag with transfer set from feeder cell media transfer pack, kept weld toward 1L transfer pack. Place “Complete CM2 Day 11 Media” prepared in incubator until use.

Day 11 – TIL Harvest

[00807] Incubator parameters: Temperature LED Display: 37.0 ± 2.0 °C; CO₂ Percentage: 5.0 ± 1.5 % CO₂. Performed check to ensure incubation parameters are met before removing G-Rex100MCS from incubator. Lower limits are the same as described above.

[00808] Record time of removal from incubator. Carefully remove G-Rex100MCS from incubator and ensured all clamps were closed except large filter line. Record processing start time.

[00809] Label a 300 mL Transfer pack as “TIL Suspension”. Sterile weld the TIL Suspension transfer (single line) of a Gravity Blood Filter. Place the 300mL Transfer Pack on a scale and record dry weight. Labeled 1L Transfer Pack as “Supernatant”.

[00810] Sterile weld the red media removal line from the G-Rex100MCS to the “Supernatant” transfer pack. Sterile weld 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. Place G-Rex100MCS on the left side of the GatheRex and the “Supernatant” and “TIL Suspension” transfer packs to the right side.

[00811] 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. Install the clear harvest line from the G-Rex100MCS to the bottom clamp (marked with a blue line) and tubing guides on the GatheRex. Attach the gas line from the GatheRex to the sterile filter of the G-Rex100MCS flask. Before removing the supernatant from the G-Rex100MCS flask, ensure all clamps on the cell removal lines were closed. Transfer ~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.

[00812] After removal of the supernatant, close all clamps to the red line.

[00813] Vigorously tap flask and swirl media to release cells. Perform an inspection of the flask to ensure all cells have detached. Tilt flask away from collection tubing and allow tumor pieces to settle along edge. Slowly tip flask toward collection tubing so pieces remained on the opposite side of the flask. If the cell collection straw is not at the junction of the wall and bottom membrane, rap the flask while tilted at a 45° angle is usually sufficient to properly position the straw.

[00814] Release all clamps leading to the TIL Suspension transfer pack. Using the GatheRex, transfer the cell suspension through the blood filter into the 300 mL transfer pack. Maintain the tilted edge until all cells and media are collected. Inspect membrane for adherent cells. Rinse the bottom of the G-Rex100MCS. Cover ~1/4 of gas exchange membrane with rinse media. Ensure all clamps are closed. Heat seal the TIL suspension transfer pack as close to the weld as possible so that the overall tubing length remains approximately the same. Heat sealed the “Supernatant”

transfer pack. Maintain enough line to weld. Record weight of TIL Suspension transfer pack and calculate the volume of cell suspension.

[00815] Weld a 4-inch (4”) plasma transfer set to “supernatant” transfer pack, retaining the luer connection on the 4-inch plasma transfer set, and transfer into the BSC. Weld a 4-inch plasma transfer set to 300 mL “TIL Suspension” transfer pack, retain the luer connection on the 4-inch plasma transfer set, and transfer into the BSC.

[00816] 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.” Remove a 1.0 mL sample from the 50 mL conical labeled BacT using an appropriately sized syringe and inoculate the anaerobic bottle.

[00817] Label 4 cryovials with vial number (1-4). Using separate 3mL syringes, pull 4x1.0mL cell count samples from TIL Suspension Transfer Pack using the luer connection, and place in respective cryovials. Placed a red cap (W3012845) on the line. Place TIL Transfer Pack in incubator until needed. Perform cell counts and calculations. Perform initial cell counts undiluted. If no dilution needed, “Sample [μ L]” = 200, “Dilution [μ L]” = 0.

[00818] Record cell counts and TIL numbers. If Total Viable TIL Cells is $< 5 \times 10^6$ cells, proceeded to “Day 11 G-Rex Fill and Seed”. If Total Viable TIL Cells is $> 5 \times 10^6$, proceed to “Calculation for flow cytometry”.

[00819] If the Total Viable TIL Cell count was $\geq 4.0 \times 10^7$, calculated the volume to obtain 1.0×10^7 cells for the flow cytometry sample. Total viable cells required for flow cytometry: 1.0×10^7 cells. Volume of cells required for flow cytometry: Viable cell concentration divided by 1.0×10^7 cells.

[00820] If Applicable: Recalculate Total Viable Cells and Volume flow. Calculate the remaining Total Viable Cells and remaining volume after the removal of cytometry sample below.

[00821] If applicable: Calculate volume for cryopreservation. Calculate the volume of cells required to obtain 1×10^7 cells for cryopreservation.

Table 24. TIL Cryopreservation Calculation.

Total Viable TIL required for cryopreservation	Viable Cell Concentration	Volume of Cells required for cryopreservation C=A÷B
A. 1×10^7 cells	B. cells/mL	C. mL

[00822] If applicable: Remove sample for Cryopreservation. Remove the calculated volume from the TIL Suspension transfer pack. Place in appropriately sized conical tube and label as “Cryopreservation Sample 1×10^7 cells,” dated, and lot number. Place a red cap (W3012845) on the TIL Suspension transfer pack.

[00823] Centrifuge the “Cryopreservation Sample 1×10^7 cells” according to the following parameters: Speed: $350 \times g$, Time: 10:00 minutes, Temperature: Ambient, Brake: Full (9); Acceleration: Full (9).

[00824] Add CS-10. In BSC, aseptically aspirate supernatant. Gently tap bottom of tube to resuspend cells in remaining fluid. Added CS-10. Slowly added 0.5 mL of CS10. Record volume added. Cryopreservation Sample Vials Filled at ~0.5 mL.

Day 11 – Feeder Cells

[00825] Obtain 3 bags of feeder cells with at least two different lot numbers from LN₂ freezer. Keep cells on dry ice until ready to thaw. Record feeder cell information. Confirm that at least two different lots of feeder cells were obtained. Place the Feeder Cell bags into individual zip top bags, based on Lot number, and thawed $37.0 \pm 2.0^\circ\text{C}$ water bath or cytoterm for ~3-5 minutes or until ice has just disappeared.

[00826] Feeder cell harness preparation. Weld 4S-4M60 to a CC2 Cell Connect (W3012820), replacing a single spike of the Cell Connect apparatus with the 4-spike end of the 4S-4M60 manifold. Weld as needed.

[00827] Attach media transfer pack: Weld the “Feeder Cell CM2 Media” transfer pack to a CC2 luer. The bag will be attached to the side of the harness with the needless injection port. Transferred the assembly containing the Complete CM2 Day 11 Media into the BSC.

[00828] Pool thawed feeder cells. Within the BSC, pull 10 mL of air into a 100 mL syringe. Used this to replace the 60 mL syringe on the CC2. Wipe 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. Maintain constant pressure while turning the spike in one direction. Do not puncture the side of the port. Opened the stopcock so that the line from the feeder cell bags is open and the line to the needless injection port is closed. Draw 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, clamp off the line to the feeder cell bags. Do not detach syringe below. the syringe from the harness. Record the total volume of feeder cells in the syringe.

[00829] Add feeder cells to transfer pack. Turn the stopcock so the line to the feeder cell bag was closed and the line to the media Transfer Pack was open. Ensure the line to media transfer pack is unclamped. Dispense the feeder cells from the syringe into the “Feeder Cell CM2 Media” transfer pack. Clamp off the line to the transfer pack containing the feeder cells and leave the syringe attached to the harness. Massage bag to mix the pooled feeder cells in the transfer pack. Labeled bag as “Feeder Cell Suspension”.

[00830] Calculated the total volume of feeder cell suspension. Remove cell count samples. Using a separate 3 mL syringe for each sample, pulled 4×1.0 mL cell count samples from Feeder Cell Suspension Transfer Pack using the needless injection port. Aliquot each sample into labeled cryovials.

[00831] Perform cell counts and calculations utilizing NC-200 and Process Note 5.14. Dilute cell count samples by adding 0.5 mL of cell suspension into 4.5 mL of AIM-V media labelled with the lot number and “For Cell Count Dilutions”. This will give a 1:10 dilution.

[00832] Record Cell Count and Sample volumes. If Total Viable Cells are $< 5 \times 10^9$, proceed. If Total Viable Cells are $\geq 5 \times 10^9$, proceed as above for higher cells counts. Obtain additional feeder cells as needed and added to transfer pack as discussed above. Calculate the volume of feeder cell suspension that was required to obtain 5×10^9 viable feeder cells. Calculate the volume of excess feeder cells to remove. Round down to nearest whole number.

[00833] Remove excess feeder cells. In a new 100 mL syringe, pull up 10mL of air and attached the syringe to the harness. Open the line to the “Feeder Cell Suspension” transfer pack. Using the syringe, draw up the volume of feeder cells calculated plus an additional 10.0 mL from the Transfer Pack into a 100 mL syringe. Close the line to the Feeder Cell Suspension transfer

pack once the volume of feeder cells is removed. Do not remove final syringe. Once a syringe has been filled, replace it with a new syringe. Multiple syringes could be used to remove total volume. With each new syringe, pull in 10mL of air. Record the total volume (including the additional 10 mL) of feeder cells removed.

[00834] Added OKT3. In the BSC, using a 1.0 mL syringe and 16 gauge (16G or 16 Ga) needle, draw up 0.15 mL of OKT3. Aseptically remove the needle from the syringe and attach the syringe to the needless injection port. Injected the OKT3. Open the stopcock to the “Feeder Cell Suspension” transfer pack and add 10 mL of feeder cells removed previously to flush OKT3 through the line. Turn the syringe upside down and push air through to clear the line to the Feeder Cell Suspension transfer pack. Leave the remaining feeder cell suspension in the syringe. Close all clamps and remove the harness from the BSC. Heat seal the Feeder Cell Suspension transfer pack, leaving enough tubing to weld.

Day 11 – G-Rex Fill and Seed

[00835] Set up G-Rex500MCS. Remove a G-Rex500MCS from packaging and inspected the flask for any cracks or kinks in the tubing. Ensure all luer connections and closures were tight. Closed all clamps on the G-Rex500MCS lines except for the vent filter line. Using a marker draw a line at the 4.5L gradation. Removed the “Complete CM2 Day 11 Media”, from the incubator.

[00836] Prepare to pump media. Weld the red line of the G-Rex500MCS to the repeater pump transfer set attached to the complete CM2 Day 11 Media. Hang the “Complete CM2 Day 11 Media” bag on an IV pole. Fed the pump tubing through the Baxa pump. Pump media into G-Rex500MCS. Set the Baxa pump to “High” and “9”. Pump 4.5L of media into the G-Rex500MCS, filling to the line marked on the flask at the 4.5L gradation. Heat seal the red line of the G-Rex500MCS near the weld. Label the flask with the “Day 11” label. Weld the Feeder Cell: Suspension transfer pack to the flask. Sterile weld the red line of the G-Rex500MCS to the “Feeder Cell Suspension” transfer pack.

[00837] Add Feeder Cells to G-Rex500MCS. Open all clamps between Feeder Cell Suspension and G-Rex500MCS and added Feeder Cell Suspension to flask by gravity feed. Heat seal the red line near the weld. Welded the TIL Suspension transfer pack to the flask. Sterile weld the red line of the G-Rex500MCS to the “TIL Suspension” transfer pack.

[00838] Add TIL to G-Rex500MCS. Open all clamps between TIL Suspension and G-Rex500MCS and added TIL Suspension to flask by gravity feed. Heat seal the red line near the weld to remove the TIL suspension bag.

[00839] Incubate G-Rex500MCS. Check 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.

[00840] Calculate incubation window. Perform calculations to determine the proper time to remove G-Rex500MCS from incubator on Day 16. Lower limit: Time of incubation + 108 hours. Upper limit: Time of incubation + 132 hours.

Day 11 – Excess TIL Cryopreservation

[00841] Freeze Excess TIL Vials. Record and verify the total number of vials placed into the Control Rate Freezer (CRF). Upon completion of freeze, transfer vials from CRF to the appropriate storage container.

Day 16 – Media Preparation

[00842] Pre-warm AIM-V Media. Remove three CTS AIM V 10L media bags from 2°C to 8°C at least 12 hours prior to use and place at room temperature protected from light. Label each bag. Record warming start time and date. Ensure all bags have been warmed for a duration between 12 and 24 hours.

[00843] Attach 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. Setup 10L Labtainer for Supernatant. Label as “Supernatant”. Setup 10L Labtainer for Supernatant. Ensure all clamps were closed prior to removing from the BSC.

[00844] Thaw 5×1.1 mL aliquots of IL-2 (6×10^6 IU/mL) (BR71424) per bag of CTS AIM V media until all ice had melted. Aliquot 100.0mL of GlutaMax into an appropriately sized receiver. Record the volume added to each receiver and labeled each receiver as “GlutaMax.”

[00845] Add IL-2 to GlutaMax. Using a micropipette, add 5.0 mL of IL-2 to each GlutaMax receiver. Rinse the tip per process and use a new pipette tip for each mL added. Record volume added to each GlutaMax receiver and labeled each receiver as “GlutaMax + IL-2” and receiver number.

[00846] Prepare CTS AIM V media bag for formulation. Ensure CTS AIM V 10L media bag (W3012717) was warmed at room temperature and protected from light for 12-24 hours prior to use. Record end incubation time. In the BSC, close clamp on a 4" plasma transfer set, then connected to the bag using the spike ports. Maintain constant pressure while turning the spike in one direction. Ensure to not puncture the side of the port. Connect the larger diameter end of a repeater pump fluid transfer set to the 4" plasma transfer set via luer.

[00847] Stage Baxa pump next to BSC. Remove pump tubing section of repeater pump fluid transfer set from BSC and installed in repeater pump.

[00848] Prepare to formulate media. In BSC, remove syringe from Pumpmatic Liquid-Dispensing System (PLDS) and discarded. Ensure to not compromise the sterility of the PLDS pipette. Connect PLDS pipette to smaller diameter end of repeater pump fluid transfer set via luer connection and placed pipette tip in "GlutaMax + IL-2" prepared above for aspiration. Open all clamps between receiver and 10L bag.

[00849] Pump 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. Record the volume of GlutaMax containing IL-2 added to each Aim V bag below.

[00850] Remove PLDS. Ensure all clamps are closed, and removed the PLDS pipette from the repeater pump fluid transfer set. Remove repeater pump fluid transfer set and red cap the 4" plasma transfer set.

[00851] Label each bag of "Complete CM4 Day 16 media" prepared.

[00852] Remove Media Retain per Sample Plan. Using a 30 mL syringe, remove 20.0 mL of "Complete CM4 Day 16 media" by attaching syringe to the 4" plasma transfer set and dispensed sample into a 50 mL conical tube. Ensure 4" plasma transfer set was either clamped or red capped after removal of syringe.

[00853] Attach new repeater pump fluid transfer set. Attach 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. Label with sample plan inventory label and stored media retain sample at 2-8°C until submitted for testing.

[00854] Monitored Incubator. If applicable, monitor for additional bags prepared. Incubator parameters: Temperature LED Display: 37.0 ± 2.0 °C, CO₂ Percentage: 5.0 ± 1.5 %CO₂.

[00855] Warmed Complete CM4 Day 16 Media. Warm the first bag of Complete CM4 Day 16 Media in incubator for ≥ 30 minutes until ready for use. If applicable, warm additional bags.

[00856] Prepared Dilutions. In the BSC, added 4.5 mL of AIM-V Media that had been labelled with “For Cell Count Dilutions” to each 4 each, 15 mL conical tube. Label the conical tubes. Labeled 4 cryovials.

Day 16 – REP Spilt

[00857] Monitor Incubator. Incubator parameters: Temperature LED Display: 37.0 ± 2.0 °C, CO₂ Percentage: 5.0 ± 1.5 %CO₂.

[00858] Remove G-Rex500MCS from Incubator. Perform check below to ensure incubation parameters are met before removing G-Rex500MCS from incubator: upper limit, lower limit, time of removal. Remove G-Rex500MCS from the incubator.

[00859] Heat seal a 1L transfer pack (W3006645), leaving ~12 inches of line. Label 1L transfer pack as TIL Suspension. Place 1L transfer pack, including the entire line, on a scale and record dry weight.

[00860] GatheRex Setup. Sterile weld the red media removal line from the G-Rex500MCS to the repeater pump transfer set on the 10L labtainer bag “Supernatant” prepared above. Sterile weld the clear cell removal line from the G-Rex500MCS to the TIL Suspension transfer pack prepared above. Place G-Rex500MCS flask on the left side of the GatheRex. Place the supernatant labtainer bag and TIL suspension transfer pack to the right side. Install the red media removal line from the G-Rex500MCS to the top clamp (marked with a red line) and tubing guides on the GatheRex. Install the clear harvest line from the G-Rex500MCS to the bottom clamp (marked with a blue line) and tubing guides on the GatheRex. Attach the gas line from the GatheRex to the sterile filter of the G-Rex500 MCS. Before removing the supernatant from the G-Rex500MCS, ensure all clamps on the cell removal lines were closed.

[00861] Volume Reduction of G-Rex500MCS. Transfer ~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.

[00862] Prepare flask for TIL Harvest. After removal of the supernatant, close all clamps to the red line.

[00863] Initiation of TIL Harvest. Record the start time of the TIL harvest. Vigorously tap flask and swirl media to release cells. Perform an inspection of the flask to ensure all cells have detached. Tilt the flask to ensure hose is at the edge of the flask. If the cell collection straw is 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 straw.

[00864] TIL Harvest. Release all clamps leading to the TIL suspension transfer pack. Using the GatheRex, transfer the cell suspension into the TIL Suspension transfer pack. Be sure to maintain the tilted edge until all cells and media are collected. Inspect membrane for adherent cells.

[00865] Rinsed flask membrane. Rinsed the bottom of the G-Rex500MCS. Cover ~1/4 of gas exchange membrane with rinse media. Closed clamps on G-Rex500MCS. Ensured all clamps were closed on the G-Rex500MCS.

[00866] Heat seal the Transfer Pack containing the TIL as close to the weld as possible so that the overall tubing length remained approximately the same. Heat seal the 10L Labtainer containing the supernatant and passed into the BSC for sample collection.

[00867] Record weight of Transfer Pack with cell suspension and calculate the volume suspension. Prepare transfer pack for sample removal. Weld a 4" plasma transfer set to the TIL Suspension transfer pack from above, leaving the female luer end attached as close to the bag as possible.

[00868] Remove 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. Place into a 15 mL conical tube and label as "BacT" and Retain the tube for BacT sample. Using a separate syringe, removed 10.0 mL of supernatant and placed into a 15 mL conical tube. Retained the tube for mycoplasma sample for testing. Label tube as "Mycoplasma diluent". Closed supernatant bag. Place a red cap on the luer port to close the bag, and pass out of BSC.

[00869] Remove Cell Count Samples. In the BSC, using separate 3 mL syringes for each sample, removed 4× 1.0 mL cell count samples from “TIL Suspension” transfer pack using the luer connection. Place samples in cryovials prepared above.

[00870] Remove Mycoplasma Samples. Using a 3 mL syringe, removed 1.0 mL from TIL Suspension transfer pack and place into 15 mL conical labeled “Mycoplasma diluent” prepared above. Label and stored Mycoplasma sample at 2-8°C until submitted for testing.

[00871] Prepare Transfer Pack for Seeding. In the BSC, attach the large diameter tubing end of a Repeater Pump Fluid Transfer Set to the Luer adapter on the transfer pack containing the TIL. Clamp the line close to the transfer pack using a hemostat. Place a red cap onto the end of the transfer set.

[00872] Place TIL in incubator. Remove cell suspension from the BSC and place in incubator until needed. Record time.

[00873] Perform cell counts and calculations utilizing NC-200. Diluted cell count samples initially by adding 0.5 mL of cell suspension into 4.5 mL of AIM-V media prepared above. This gave a 1:10 dilution.

[00874] Calculate flasks for subculture. Calculate the total number of flasks to seed. Rounded the number of G-Rex500MCS flasks to seed up to the neared whole number.

Example 6. Small-scale Production of TILs from Cryopreserved Tumor Tissue.

[00875] Preparing the tumor tissue for freezing: The tumor was aseptically collected, trimmed to remove extraneous non-tumor or necrotic tissue using sterile tweezers and a scalpel and/or scissors. Tumors with the minimum size of 1.5 cm diameter were further fragmented into 6 mm size fragments. For freezing the tumor, up to 10 pieces of 6 mm diameter fragments were placed in a single 15 mL cryovial (Thermo, USA) containing 10 mL of CryoStor10 (Biolife, USA) storage medium. Additional cryovials were used if the 6 mm diameter fragments exceeded 10 fragments. Cryovial lids were screwed tightly; then the tumor fragments were mixed gently with the storage medium by iteratively inverting the cryovial at least 3 and up to about 5 times. Then, the cryovials were incubated for 15 to 60 minutes at 2°C-8°C in a refrigerator. After incubation, cryovials slotted into a cryoshipper sleeve.

[00876] Freezing the tumor tissue: The cryoshipper sleeve containing the cryovials was placed in into a liquid nitrogen dry cryoshipper, thereby flash freezing the contents of the vials using the vapor phase liquid nitrogen temperature inside the LN₂ charged and temperature equilibrated dry LN₂ cryoshipper. For short term storage or transport, vials remained in the cryoshipper. For long term storage, vials were transferred and stored at liquid nitrogen temperatures in a liquid nitrogen freezer.

[00877] Thawing the stored tumor tissue: Frozen tumor samples were thawed by submerging the cryovials in a 37°C water bath for 5 ± 1 min. Using forceps, 6 mm diameter fragments were removed, washed three times with sterile Hank's Balanced Salt Solution (HBSS) (Gibco, USA) with gentamicin (Gibco, USA). Gentamicin was added to the HBSS at 50 µg/mL. Each 6 mm diameter fragment were further dissected to approximately 8 individual 1 to 3 mm diameter fragments. Typical tumor fragments were about 1 to 3 mm diameter and about 1 to 3 mm thick.

[00878] TIL Expansion, using a 1/100th Scale procedure: This procedure replicates the full-scale process 2A TIL Manufacturing method on a 1/100th scale. The scope of the study is to give instruction for the miniaturized 2A process using G-Rex 10M and G-Rex 5M flasks. PreREP culture was initiated using G-Rex 10M flasks, 1:10 of the full-scale pre-REP process. The REP was initiated using 10% of the pre-REP product in G-Rex 5M which equates to 1:10 of the full-scale REP process. This maintained a 1:100 scale for the remainder of the experiment.

[00879] The pre-REP was initiated with tumor processing and four fragments placed in an individual G-Rex 10M flask and cultured with CM1+IL-2 until Day 11. The REP was initiated on Day 11 using pre-REP TIL (10% of pre-REP product), PBMC feeders, OKT3 and IL-2 into a G-Rex 5M. Day 16 will have retained the appropriate amount of TIL and a multiplication factor will be used to extrapolate the full-scale process. Figure 7 summarizes the proecedure employed for this experimental scale TIL production.

[00880] Table 25 lists the equipment used for this experimental scale TIL production.

TABLE 25. Equipment.

Item	Specifications	Company	Model #	Calibration requirements
Incubator	Set up at 37°C, 5%CO ₂	Thermo Scientific	Forma Steri-Cycle i160	Temperature is monitored every week.
Centrifuge	With swinging bucket rotor	Thermo Scientific or equivalent	Sorvall Legend XTR-7500-4521	NA
Centrifuge	Microcentrifuge table top	Eppendorf or equivalent	NA	NA
Flow cytometer	Equipped with 3 lasers (red, blue, violet)	BD Biosciences	BD FACS Canto II	Yearly Preventive Maintenance. CST Baseline and daily performance
Vortex Mixer	NA	Labnet International or equivalent	NA	NA
Pipet Aid	NA	Drummond or equivalent	NA	NA
2 - 20-µL pipet	NA	Rainin or equivalent	L-20XLS or equivalent	Every six months
20 - 200-µL pipet	NA	Rainin or equivalent	L-200XLS or equivalent	Every six months
100 - 1000-µL pipet	NA	Rainin or equivalent	L-1000XLS or equivalent	Every six months
Biological Safety Cabinet (BSC)	NA	Thermo Scientific or equivalent	NA	Every six months
Water Bath	Set and maintain at 37°C +/- 2 degrees	Any in use	NA	NA
Cell counter	Dual fluorescence	Nexcelom or equivalent	K2	As recommended by vendor
Refrigerator	Set and maintain at 4°C +/- 2 degrees	Any in use	NA	NA
Freezer	Set and maintain at -20°C +/- 5 or 4 °C degrees	Any in use	NA	NA

[00881] Table 26 lists the software used for this experimental scale TIL production.

TABLE 26. Software.

Name	Company	Version
FACSDiva	BD Biosciences	7 or equivalent / upgraded
FlowJo	Flow Jo	10
Cellometer K2	Nexcelom	2.1.4.2

[00882] Table 27 lists the materials used in this experimental scale TIL production.

TABLE 27. Materials.

Product	Specifications	Vendor	Catalog #	Storage
50-mL conical tubes	NA	VWR or equivalent	89004-364	RT
G-Rex 5M	NA	Wilson Wolf	NA	RT
G-Rex 10M	NA	Wilson Wolf	NA	RT
10-ml pipet	NA	Denville or equivalent	P7134	RT
5-mL pipet	NA	Fisher Scientific	13-678-11D	RT
25-mL pipet	NA	Fisher Scientific	13-678-11	RT
50-mL pipet	NA	Fisher Scientific	13-675-27	RT
20-μL pipet tips	NA	VWR or equivalent	1011-260-000	RT
200-μL pipet tips	NA	VWR or equivalent	1018-260-000	RT
1000-μL pipet tips	NA	VWR or equivalent	1019-260-000	RT
10-mL pipet	NA	VWR or equivalent	89130-898	RT
Slides for cellometer	NA	Nexcelom Biosciences	SD100	RT
FACS tubes	NA	BD Biosciences	343675	RT
Microfuge tubes	1.5 ml	Eppendorf or equivalent	022363328	RT
Aluminum Foil	NA	VWR or equivalent	N/A	RT
BD FACS Flow Sheath Fluid	NA	BD Biosciences	342003	RT
500 mL Stericup Filter system	NA	Millipore	SCGPU05RE	RT
1X D-PBS	Without Ca ²⁺ /Mg ²⁺	Sigma or equivalent	D-8537	RT
BD Cytometer Setup and Tracking Beads	NA	BD	655051	4°C
BD FACS Clean solution	NA	BD	340345	RT
BD Shutdown solution	NA	BD	334224	RT

[00883] Table 28 lists additional materials and reagents used.

TABLE 28. Additional Materials and Reagents.

Product	Specifications	Vendor	Catalog #	Storage
BD Stain buffer	Dulbecco's Phosphate-Buffered Saline (DPBS) with 2% (w/v) heat-inactivated FBS and 0.09% (w/v) sodium azide, pH 7.4 (0.2 μ m-pore filtered)	BD Pharmingen	554656	4°C
10% Bleach in Distilled water	Any in use	NA	NA	RT
Distilled water	Any in use	NA	NA	RT
FACS tube racks	Any in use	NA	NA	RT
Gloves, Latex / Nitrile	Any in use	NA	NA	RT
Lint free wipes	Any in use	NA	NA	RT
70% Ethanol	Any in use	NA	NA	RT
Paper towels	Any in use	NA	NA	RT
CaviCide	NA	Fisher or equivalent	22-998-805	RT
CryoStor 10	NA	Stemcell Technologies	07930	4°C

[00884] Table 29 lists reagents used for flow cytometry (FACS) analysis of cells to characterize various marker expression.

TABLE 29. FACS Reagents.

Flow reagents			
Marker	Fluorochrome	Vendor	Catalog #
Live/dead Aqua	Aqua	Life Technologies	L34957
CD4	FITC	eBioscience	11-0037-42
CD8	Pacific Blue	Biologend	301033
TCR α/β	PE	Biologend	306708
CD57	PerCP/Cy5.5	Biologend	359622
CD27	APC H7	BD Biosciences	560222
CD28	PECy7	BD Biosciences	560684
CD56	APC	Beckman	IM2474U

[00885] Table 30 lists reagents used for cell culture media.

TABLE 30. Media Reagents

Product	Specifications	Vendor	Catalog #	Storage
RPMI	Contains: L-glutamine and Phenol red. Without: HEPES	Life Technology	11875-119	4°C
CTS Optimizer	-L-Glutamine Phenol Red	Life Technologies	A1048501	4°C
Serum replacement	L-Glutamine	Life Technologies	A2596101	-20°C
Human AB Serum	GemCell™ human serum AB is collected from healthy male donors of the AB serotype at FDA-licensed facilities in the United States. 0.1µm sterile-filtered	Gemini Bio-Products	100-512	-20°C
L-Glutamine	NA	Lonza	BW17-605E	-80°C
Gentamycin	NA	Gibco/ Life Technology	15710-064	RT
2-Mercaptoethanol	NA	Gibco/ Life Technology	21985-023	4°C
GMP Recombinant Human Interleukin-2 (rhIL-2), 8e6 stock solution	NA	CellGenix	1020-1000	-20°C
AIM-V Medium CTS	NA	Gibco/Life Technologies	087-0112DK	4°C
GlutaMax Supplement	NA	Gibco Life Technology	35050-061	RT
AO/PI dye	NA	Nexcelom	CS2-0106-5ml	4°C

*Procedure for mini-scale TIL expansion (1/100th scale 2A Process)**Day 0*

[00886] Media Preparation, if necessary, prepare IL-2. Prepare 350 mL CM1 for every 3 G-Rex 10M flasks. Add 6000 IU/mL IL-2 to CM1. Warm in 37°C water bath while processing tumor.

[00887] Tumor Processing: Add 100mL of media and IL-2 to each G-Rex 10M flasks and randomly add 4 fragments to each flask. Be sure to randomize fragments to increase heterogeneity of results. Place in the incubator at 37°C/5% CO₂ for 11 days.

Day 11

[00888] Media Preparation, if necessary, prepare IL-2. Prepare 300 mL CM2 for every 3 G-Rex 5M flasks. Add 3000 IU/mL IL-2 to CM2, then warm in 37°C water bath.

[00889] Prepare the TIL from first culture: Carefully remove each G-Rex 10M flask from incubator without disturbing cells. Aspirate media and using a 10 mL pipette, gently mix up and

down to liberate cells from membrane. Slowly pull culture up and record volume from each flask, transfer to a 50 mL conical vial.

[00890] Obtain a 200 μ L aliquot for cell counts, then loosely cap the 3 vials and place in 37°C/5% CO₂ incubator until ready to seed flasks. Count TIL and record on cell counting worksheet.

[00891] Prepare feeder cells: Obtain an adequate number of feeders (50×10^6 cells/flask); thaw feeder cells in 37°C water bath for 2 minutes, then dilute feeder cells 1:10 in CM2+IL-2. Remove a 200 μ L aliquot for cell counting, loosely cap vial and place in 37°C/5% CO₂ incubator until ready to seed flasks. Dilute 20uL PBMC feeder cells 1:10 and count PBMC feeders. Record on cell counting worksheet. Based on the count, dilute feeders to 10×10^6 cells/mL in CM2+IL-2, then place 5 mL of solution into a 50 mL conical tube for each flask. For each flask, add 1.5 mL of 1:1000 diluted stock OKT-3 to the 5mL of feeders and incubate for 15 minutes.

[00892] Seed flasks by adding TIL, PBMC feeder cells, OKT-3, and media to each flask. For TILs, add about 10% of the preREP produce, generally up to a maximum of about 2×10^6 cells per flask. PBMC feeders, add 5 mL prepare as above, containing 50×10^6 cells total. Add 1.5 mL of 1:1000 diluted OKT-3 stock, then bring to a final volume of 50 mL by topping with CM2 medium.

Day 16

[00893] Media preparation for the split: If necessary, prepare IL-2; then prepare 150 mL CM4 for every 3 flasks. Next, add 3000 IU/mL IL-2 to media. Place in 37°C water bath until needed.

[00894] Split: Carefully remove G-Rex 5M flasks from incubator as not to disturb cells, then aspirate approximately 40 mL media, taking care to not aspirate cells. Using a 10 mL pipette, gently mix up and down to liberate cells from membrane; then, slowly pull culture up and record volume from each flask, transfer to a 50 mL conical vial. Take a 200 μ L aliquot for cell counts, and then loosely cap the 3 vials, placing them in 37°C/5% CO₂ incubator until ready to seed flasks. Count TIL and record on cell counting worksheet. Aspirate remaining liquid from bottom of G-Rex 5M flasks to ensure no extra TIL remain in flasks.

[00895] Calculate number of daughter flasks for each flask (TVC/ 10^6), round up (max 5). Divide TVC by number of daughter flasks and adjust flask to the calculated amount. Some

experiments may allow only one flask to be continued. This will also be extrapolated and accounted for at the end of the harvest. Fill flasks to 50 mL with CM4+IL2 and then place flasks in 37°C/5% CO₂.

Day 22

[00896] Harvest: Carefully remove G-Rex 5M flask from incubator as not to disturb cells, then remove supernatant, if needed for metabolite analysis. Aspirate media and using a 10 mL pipette, gently mix up and down to liberate cells from membrane. Slowly pull culture up and record volume from each flask, then transfer to a 50 mL conical vial. Remove a 200 µL aliquot for cell counts; loosely cap the 3 vials and place in 37°C/5% CO₂ incubator until ready to seed flasks.

[00897] Count TIL record on cell counting worksheet; extrapolate and record theoretical full-scale yield (TVC*100). If only one flask is continued in split, also multiply by # of flasks calculated above. Then, allocate the cells for the assays listed in Table 31.

TABLE 31. Functional Assays.

Functional Assay	Number of Cells/Culture Supernatant
Flow Phenotyping (DF1/DF2) – WRK LAB-041	≥10e6
IFN-γ Re-stimulation -WRK LAB-016	≥5e6
Cells to freeze	All Remaining cells

[00898] Wash remaining cells two times with a 1:10 dilution of 1% HSA/PL-A. Freeze remaining cells in a thermally-insulated container (such as a Mr. Frosty container) or CRF in a 1:1 formulation with CS10 (30 × 10⁶ cells/mL/Vial) for other studies.

[00899] Table 32 summarizes the critical process parameters for the TIL production process.

TABLE 32. Process Parameters.

IL-2 Concentration	CM1-6000IU/mL CM2/CM4-3000IU/mL
OKT-3 Concentration	Day 11 – 30ng/mL
Feeder seeding density	Day 11 – 50e6/flask
TIL seeding density	Day 11 - ≤2e6/flask (10% of pre-REP product)
Preloading of OKT-3	15 Minutes incubation of OKT-3 and feeder cells
TIL seeding density	Day 16 - Divide evenly across calculated daughter flasks
Calculation of Daughter flasks	Day 16 TVC / 1e9, maximum 5.
Media Preparation	Properly prepared according to LAB-005
Product Formulation	2X wash with 1% HSA/PL formulated with 1:1 TIL/CS10 ratio

Example 7. Early REP with Frozen Tumor Tissue TILs.

[00900] This data was generated using a modified version of the TIL manufacturing protocol detailed in Example 5. Aspects of the experimental method that differ from Example 5 are highlighted.

[00901] Tumor tissue preparation: Care was taken to keep the tumor hydrated by adding Hank's Balanced Salt Solution (HBSS) to the tissue as needed, keeping it hydrated through drop-wise addition. The tumor was trimmed to remove extraneous non-tumor tissue using sterile tweezers, a scalpel, and scissors. Tumor fragments with a minimum size of 1.5 cm diameter were further fragmented into 6 mm diameter pieces. Ideal thickness for each fragment was roughly 6 mm

[00902] Tumor tissue freezing: Up to ten tumor fragments (nominal size 6 mm × 6 mm × 6 mm) were placed in each cryovial. 15 mL cryovials (Thermo, USA) were used. 10 mL of CryoStor10 (Biolife, USA) was added to each tube. Cryovial lids were screwed tightly and the contents of the cryovial were mixed gently by inverting the tube 5 times. The cryovials were then incubated at 2°C-8°C in a refrigerator, for 15 minutes, 30 minutes, or 60 minutes. After the

refrigerated incubation in storage medium, cryovials were packed in a sleeve, then flash frozen using the vapor phase of liquid nitrogen (LN₂) in a properly charged and equilibrated dry liquid nitrogen shipper (Cryoport). For storage, fully frozen cryovials were transferred to a liquid nitrogen cell storage freezer, to maintain the frozen tumor tissue at liquid nitrogen temperatures.

[00903] Tumor thawing: On Day 0 of the TIL manufacturing process, cryovials containing the tumor tissue fragments were removed from the sleeve, placed in a sealable plastic bag, then thawed by submerging the sealed bag in a 37°C water bath for 5 ± 1 minute.

[00904] By using forceps, tumor fragments were carefully removed into sterile HBSS (Gibco, USA) + Gentamicin (Gibco, USA), serially washed 3 times in HBSS containing gentamicin (50 µg/mL) for ≥ 3 minutes each wash. Individual fragments approximately 5-6 mm in all dimensions were cut from the original tumor samples and staged in individual drops of HBSS on a new sterile plastic surface.

[00905] Up to 60 of these fragments were placed into a G-Rex 100MCS flasks containing 1 L of Cell Culture Medium 1 (CM1), supplemented with 6000 IU/mL recombinant human IL-2, and the flask was placed in a humidified incubator (target 37 °C, 5% CO₂ in air) to initiate the pre-Rapid Expansion Phase (pre-REP) unit operation.

[00906] Summary of TIL manufacture: PreREP and REP. A small-scale Gen 2 process (1/100th scale) was used to test the “fresh” control and the several frozen tumor conditions. Pre-REP culture was initiated using G-Rex 10M flasks, at 1:10 scale of the full-scale pre-REP process. REP was initiated using 10% of the pre-REP product in a G-Rex 5M, which equates to 1:100 of the full-scale REP process.

[00907] For “fresh” control process, fresh tumor was washed in sterile HBSS+ Gentamicin, sectioned to nominal 6 mm × 6 mm × 6 mm fragments. About 4 fragments were placed into a G-Rex 10M flask with CM1 media containing 6,000 IU/mL of rhIL-2. G-Rex cultures were incubated for 11 days in a 37°C incubator. On day 11 of the culture, pre-REP cells were harvested, and the REP was initiated by coculturing 10% of the Pre-REP TIL with 50×10^6 PBMC feeder cells in CM2 media containing 3,000 IU/mL rhIL-2 and 30 ng/mL of OKT-3 in a G-Rex 5M flask. On day 16 of the co-culture, the culture was split into G-Rex 5M flasks containing no more than 10×10^6 cells. The calculation of the number of split “daughter” flasks was performed as follows: $\text{TVC}/10^6 = \text{number of Daughter flasks}$, rounded up.

[00908] The split cultures were incubated for 6 additional days in CM4 media containing 3,000 IU/mL rhIL-2. On day 22, the cells were harvested and frozen using CryoStor10 (Biolife, USA).

[00909] For frozen tumor expansion process, the nominal 6 mm × 6 mm × 6 mm fragments were processed into smaller 2 mm – 3 mm fragments as explained above. Pre-REP setup, REP initiation, Split process, and reagents used for frozen tumor expansion were performed exactly like the “fresh” control process, with the following exceptions:

[00910] The duration of the Pre-REP culture was 7 days (vs 11 days for “fresh” control)

[00911] The duration of the REP culture was 14 days with a Split on Day 7 (vs 11 days with a Split on Day 5 for “fresh” control). Thus, the total duration of the frozen tumor was 21 days, vs 22 days for the “fresh” control.

[00912] Characterization or REP TILs: TILs produced from each of the tumor processing conditions (fresh, frozen with 0, 30 minutes, or 60 minute refrigerated incubations in storage medium). To characterize TIL purity, identity, memory subset, activation, and exhaustion markers, flow cytometry was conducted with the following antibodies: CD3-BUV395, CD62LBV421, CD57-PB, CD11c-BV711, CD28-BB515, CD19-BUV563, CCR7-PE, CD123-BV605, CD27PE-CF594, CD14-BV-650, TCR γ/δ -APC, CD45-PerCP-Cy5.5, CD45RA-A700, CD56-BUV737, CD8-BV786, CD4-PE-Cy7AND CD16-APC-Vio770) and TIL-2 panel (CD3-BUV395, PD-1-BV421, 2B4/CD244-PB, CD8-BB515, CD25-BUV563, BTLA-PE, KLRG1-PE-Dazzle 594, TIM-3-BV650, CD194/CCR4-APC, CD4-VioGreen, TIGIT-PerCP-eFluor 710, CD183-BV711, CD69-APC-R700, CD95-BUV737, CD127-PE-Cy7, CD103-BV786, LAG-3-APC-eFluor 780. TIL function was further determined based on secretion of IFN- γ , Granzyme-B and CD107A. Briefly, about 10⁵ cells were stimulated with anti-CD3 coated plate for 24 h. Culture supernatant was collected and tested using ELISA for IFN- γ and Granzyme-B. For CD107A levels, TIL were stimulated with or without stimulation of PMA/IO for 2 h, stained for CD107A-APC700, and further analyzed by flow cytometry.

[00913] Flow-FISH was performed using Dako/Agilent Pathology Solutions Telomere PNA Kit/FITC for Flow Cytometry kit to determine telomere length, following the manufacturer’s instructions. 1301 T-cell leukemia cell line (Sigma-Aldrich) was used as the internal reference standard.

Results

[00914] Figure 8 shows the pre-REP total viable cells (TVC) based on the tumor tissue freezing procedure. Incubation in the storage medium for about 30 minutes under refrigerated conditions (2°C - 8°C) yielded the most viable cells, compared both to immediate flash freezing and longer incubations before freezing. Figure 9 shows the REP TVC results at day 21. The freezing protocol affects the early viability in pre-REP culture. Further function was assessed by measuring IFN- γ secretion, and is summarized in Figure 10. TILs expanded from frozen tumor tissue first incubated for 30 minutes in storage medium expressed the highest level of IFN- γ . All markers evaluated in the cell phenotype panel (*see* Table 29) demonstrate that TILs produced from frozen tumor tissue have similar phenotypes as TILs produced from fresh tumor tissue.

[00915] Figures 11 and 12 overview the major steps and highlights the differences between the cell populations compared in this exemplary experiment.

[00916] Figures 13-23 illustrate the results of the TIL phenotypic characterization of fresh and frozen tumor samples in 3 to 5 patients. Patient M1125 was tested at flash freezing and 30 minute and 60 minute incubation at 2°C to 8°C prior to freezing in the vapor phase of liquid nitrogen, and all other patients were tested at the 60 minute incubation at 2°C to 8°C prior to freezing in the vapor phase of liquid nitrogen. Overall, frozen tumor samples performed as well as or better than fresh tumor samples.

Example 8. Tumor Cryopreservation Process.

[00917] Tumors may be cryopreserved in an exemplary embodiment of the present invention according to the following process. Tumor tissue must be handled aseptically and remain free of contamination throughout processing.

[00918] After the tissue is removed from the patient it should be kept hydrated by adding Hank's Balanced Salt Solution (HBSS) to the tissue as needed through drop-wise addition with a transfer pipette. The tumor is aseptically placed in a sterile specimen container with a lid and transferred to a sterile surface in the surgical suite or a laminar flow hood if sent to pathology for prosection. Using sterile tweezers and a sterile scalpel or scissors, the tumor should be trimmed to remove extraneous non-tumor or necrotic tissue by the resecting physician or an experienced pathologist designated at each site. Hemorrhagic, liquid and necrotic tissue should be excluded whenever possible. The physician or pathologist should evaluate the prosected

specimen to ensure tissue is viable, non-hemorrhagic, free of necrosis, and that the minimum size specification is met. Tissue should have pigment heterogeneity if possible.

[00919] If the tumor has a diameter of at least 1.5 cm, it may be cut in half to provide both a frozen specimen and a fresh specimen for comparison. One half of the trimmed tumor is to be placed into a sterile bottle of Hypothermosol that has been maintained at 4-8°C with gentamicin and amphotericin added. The lid should be screwed tightly. This provides the fresh specimen.

[00920] The other half of the tumor should be fragmented into 10 x 6 mm fragments and placed into a prepared sterile 15 mL cryovial containing 10 mL of sterile CryoStor® 10. If more than ten 6 mm fragments are obtained, use additional 15 mL cryovial containing 10 mL of sterile CryoStor 10. The lid should be screwed tightly and the frozen specimen container should be placed at 4°C for 1 hour and then flash frozen over liquid nitrogen. This provides the frozen specimen.

[00921] The frozen tumor specimen should be packaged into a cryoshipper per cryoshipper instructions.

Example 9. Sixteen Day Frozen Tumor Expansion Processes from Ovarian Cancer Tumors.

[00922] A small scale TIL expansion process (1/100th scale) was used to test “fresh” control tumor samples and frozen tumor samples. Frozen tumor samples were frozen according to the cryopreservation process discussed above in Examples 7 and 8. Pre-REP was initiated using G-Rex 10M flasks at 1:10 of the full-scale pre-REP process. REP was initiated using 10% of the pre-REP product in a G-Rex 5M flask, which equates to about 1:100 of the full-scale REP process. Ovarian tumor samples were used in this example.

[00923] Fresh Tumor Preparation. Six 3 mm diameter fresh tumor fragments were washed in sterile Hanks Balanced Salt Solution (HBSS) supplemented with gentamicin. The fragments were placed into a G-Rex 10M flask (Wilson Wolf Mfg., New Brighton, MN) with CM1 media containing about 6,000 IU/mL of rhIL-2. These cultures were incubated for 11 days in a 37°C incubator. On day 11, the pre-REP cells were harvested, and the REP was initiated by co-culturing 10% of the pre-REP TILs with 50x10⁶ PBMC feeder cells in CM2 media containing 3,000 IU/mL rhIL-2 and 30 ng/mL of OKT-3 in a G-Rex 5M flask (Wilson Wolf

Mfg., New Brighton, MN). On day 16, the culture was split into G-Rex 5M flasks containing no more than 10×10^6 cells. The split cultures were incubated for 6 additional days in CM4 media containing 3,000 IU/mL rhIL-2. On day 22, the cells were harvested and frozen using CryoStor10 (Biolife, USA).

[00924] Frozen Tumor Preparation – Early REP Method 1 (“ER-1”). Six 2-3 mm diameter frozen tumor fragments were thawed and washed in sterile Hanks Balanced Salt Solution (HBSS) supplemented with gentamicin. The fragments were placed into a G-Rex 10M flask (Wilson Wolf Mfg., New Brighton, MN) with CM1 media containing about 6,000 IU/mL of rhIL-2. These cultures were incubated for 5 days in a 37°C incubator. On day 5, the pre-REP cells were harvested, and the REP was initiated by co-culturing 10% of the pre-REP TILs with either 25×10^6 or 50×10^6 PBMC feeder cells in CM2 media containing 3,000 IU/mL rhIL-2 and 30 ng/mL of OKT-3 in a G-Rex 5M flask (Wilson Wolf Mfg., New Brighton, MN). On day 10, the culture was split into G-Rex 5M flasks containing no more than 10×10^6 cells. The split cultures were incubated for 6 additional days in CM4 media containing 3,000 IU/mL rhIL-2. On day 16, the cells were harvested and frozen using CryoStor10 (Biolife, USA).

[00925] Frozen Tumor Preparation – Early REP Method 2 (“ER-2”). Six 2-3 mm diameter frozen tumor fragments were thawed and washed in sterile Hanks Balanced Salt Solution (HBSS) supplemented with gentamicin. The fragments were placed into a G-Rex 10M flask (Wilson Wolf Mfg., New Brighton, MN) with CM1 media containing about 6,000 IU/mL of rhIL-2. These cultures were incubated for 3 days in a 37°C incubator. On day 3, the pre-REP cells were harvested, and the REP was initiated by co-culturing 10% of the pre-REP TILs with either 25×10^6 or 50×10^6 PBMC feeder cells in CM2 media containing 3,000 IU/mL rhIL-2 and 30 ng/mL of OKT-3 in a G-Rex 5M flask (Wilson Wolf Mfg., New Brighton, MN). On day 9, the culture was split into G-Rex 5M flasks containing no more than 10×10^6 cells. The split cultures were incubated for 7 additional days in CM4 media containing 3,000 IU/mL rhIL-2. On day 16, the cells were harvested and frozen using CryoStor10 (Biolife, USA).

[00926] Figure 24 illustrates a flow chart comparing these three expansion methods. Results are illustrated below in Table 33. Expansion of TILs using both ER-1 and ER-2 methods produced a much larger REP fold expansion as compared with using the fresh tumor sample method GEN2.

Table 33: Expansion Results from Example 9 Extrapolated to Full Scale.

	Gen2	ER-1 Frozen	ER-1 Frozen with half feeders	ER-2 Frozen	ER-2 Frozen with half feeders
Day 0 No. of fragments/flasks	60	60	60	60	60
Day 11 – Pre-REP TVC (% viability)	2.93x10 ⁶ (88.7)	1.23x10 ⁶ (64)	1.23x10 ⁶ (64)	6.1x10 ⁵ (55)	6.1x10 ⁵ (55)
Day 11 REP Seeded (TIL)	2.93x10 ⁶	1.23x10 ⁶	1.23x10 ⁶	6.1x10 ⁵	6.1x10 ⁵
Day 11 REP Seeded (Feeders)	5x10 ⁹	5x10 ⁹	2.5x10 ⁹	5x10 ⁹	2.5x10 ⁹
Split Day Scale up TVC (% Viability)	Day 16 1.49 x10 ⁹ (83.2)	Day 10 1.66 x10 ⁹ (91.2)	Day 10 1.2 x10 ⁹ (91.4)	Day 9 2.17 x10 ⁹ (92)	Day 9 1.23 x10 ⁹ (86.8)
Harvest Day TVC (% viability)	Day 22 10.9 x10 ⁹ (92.9)	Day 16 29.1 x10 ⁹ (88.3)	Day 16 25.9 x10 ⁹ (89)	Day 16 49.8 x10 ⁹ (92.3)	Day 16 34.2 x10 ⁹ (89.6)
REP fold expansion	3728	23714	21060	81684	55998

[00927] Example 10: Sixteen Day Frozen Tumor Expansion Processes with Other Tumor Types.

[00928] The processes disclosed in Example 9 are performed with tumor samples collected from at least two patients in the following cancer types: melanoma, lung, cervical, and head and neck squamous cell carcinoma (HNSCC). The expansion processes are performed for each cancer type in substantially the same manner as described above for the ER-1 and ER-2 methods. Data are collected as described above.

[00929] Example 11: Sixteen Day Frozen Tumor Expansion Processes with Defined Medium.

[00930] The processes disclosed in Example 9 are performed with tumor samples collected from at least two patients in the following cancer types: melanoma, lung, cervical, head and neck squamous cell carcinoma (HNSCC), and ovarian, but substituting the CM1 and CM2 media with a defined medium according to the present invention (e.g., CTSTTM OpTmizerTM T-Cell Expansion SFM with 3% CTSTTM Immune Cell Serum Replacement (ThermoFisher Scientific) and 2-mercaptoethanol at 55mM).

[00931] Example 12: Sixteen Day Frozen Tumor Expansion Processes with OMNI C3™ culture bags and EXP-PAK® Bio-Containers.

[00932] The processes disclosed in Example 9 are performed with tumor samples collected from at least two patients in the following cancer types: melanoma, lung, cervical, head and neck squamous cell carcinoma (HNSCC), and ovarian, but substituting the GRex gas permeable containers with either the OMNI C3™ culture bags (Lampire Biological Laboratories) or the EXP-PAK® Cell Expansion Bio-Containers (Charter Medical). The OMNI C3™ culture bag is substituted at the pre-REP/first expansion stage, and the EXP-PAK® bio-container is substituted at the REP/second expansion stage.

[00933] Example 13: Sixteen Day Frozen Tumor Expansion Processes with Defined Medium, OMNI C3™ culture bags and EXP-PAK® Bio-Containers.

[00934] The processes disclosed in Example 9 are performed with tumor samples collected from at least two patients in the following cancer types: melanoma, lung, cervical, head and neck squamous cell carcinoma (HNSCC), and ovarian, but substituting the CM1 and CM2 media with a defined medium according to the present invention (e.g., CTST™ OpTmizer™ T-Cell Expansion SFM, ThermoFisher) and substituting the GRex gas permeable containers with either the OMNI C3™ culture bags (Lampire Biological Laboratories) or the EXP-PAK® Cell Expansion Bio-Containers (Charter Medical). The OMNI C3™ culture bag is substituted at the pre-REP/first expansion stage, and the EXP-PAK® bio-container is substituted at the REP/second expansion stage.

CLAIMS

We claim:

1. A method of cryopreserving tumor tissue for the manufacture of tumor infiltrating lymphocytes (TILs), the method comprising:
 - (i) fragmenting the tumor tissue;
 - (ii) incubating the fragments in a cryopreservation medium; and,
 - (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.
2. The method of claim 1, wherein the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 6 mm.
3. The method of claim 2, wherein the approximately spherical fragments have a diameter of about 6 mm.
4. The method of claim 2, wherein the approximately spherical fragments have a diameter of about 3 mm.
5. The method of claim 1, wherein the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 1.5 mm and a longest edge length of about 6 mm.
6. The method of claim 1, wherein the tumor tissue is fragmented into generally cubical fragments having edge lengths of between about 1.5 mm and about 6 mm.
7. The method of claim 6, wherein the generally cubical fragments have edge lengths of about 6 mm.
8. The method of claim 6, wherein the generally cubical fragments have edge lengths of about 3 mm.
9. The method of claim 1, wherein the tumor tissue is from a dissected tumor.

10. The method of claim 1, wherein the dissected tumor is less than eight hours old.
11. The method of claim 1, wherein the cryopreservation medium comprises 2% v/v to 15% v/v dimethyl sulfoxide (DMSO).
12. The method of claim 11, wherein the cryopreservation medium comprises about 10% v/v DMSO.
13. The method of claim 1, wherein the cryopreservation medium comprises at least one antimicrobial agent.
14. The method of claim 13, wherein the at least one antimicrobial agent is gentamicin at a concentration of at least 50 µg/mL.
15. The method of claim 1, wherein the tumor fragments are incubated in the cryopreservation medium for about 30 minutes to about 80 minutes.
16. The method of claim 1, wherein the tumor fragments are incubated in the cryopreservation medium at a temperature of about 2° to 8°C.
17. The method of claim 1, wherein the tumor tissue is washed in a physiologically buffered isotonic saline solution prior to incubation.
18. The method of claim 17, wherein the washing comprises three serial washes of at least three minutes each, with the physiologically buffered isotonic saline solution replaced after each serial wash.
19. The method of claim 1, wherein the freezing takes place at a temperature in the range from of -125°C to about -196°C.
20. The method of claim 19, wherein the freezing takes place at a temperature of about -140°C to about -175°C.
21. The method of claim 20, wherein the freezing takes place at a temperature of about -145°C.

22. The method of claim 1, further comprising the step of (iv) storing the frozen fragments below at least -130°C.
23. The method of claim 22, wherein the frozen fragments are stored in the vapor phase of liquid nitrogen.
24. The method of claim 22, wherein the frozen fragments are stored submerged in liquid nitrogen.
25. A cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by a process comprising the steps of:
- (i) fragmenting a dissected tumor or tumor biopsy sample;
 - (ii) incubating the fragments in a cryopreservation medium; and,
 - (iii) freezing the fragments wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.
26. The cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by the process of claim 25, wherein step (ii) further comprises incubating the fragments for about 30 minutes to about 60 minutes at about 2°C to about 8°C in a cryopreservation medium comprising 10% v/v DMSO.
27. A cryopreserved tumor fragment for the manufacture of tumor infiltrating lymphocytes (TILs), prepared by a process comprising the steps of:
- (i) fragmenting a dissected tumor, wherein the fragments are generally cubical shaped and about 6 mm on each edge;
 - (ii) incubating the fragments in a cryopreservation medium, the cryopreservation medium comprising 10% DMSO v/v, wherein the incubating is performed for about 30 minutes to about 60 minutes at a temperature of about 2°C to about 8°C; and
 - (iii) freezing the fragments, wherein the freezing is flash freezing using the vapor phase of liquid nitrogen.

28. A method for manufacturing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy, the method comprising culturing a cryopreserved tumor fragment in a culture medium comprising IL-2.
29. A method for preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy, the method comprising:
- (a) treating a cryopreserved tumor fragment in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;
 - (b) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs; and
 - (c) optionally cryopreserving the expanded number of TILs.
30. A method for preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy, the method comprising:
- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature of about 2 to about 8°C for a period of about 30 minutes to about 60 minutes;
 - (iv) freezing the vessel, wherein the freezing is flash freezing using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at or below -130°C;
 - (b) thawing the vessel;

- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 to provide TILs;
 - (d) expanding the TILs in a gas permeable container using a second cell culture medium comprising cell culture medium, irradiated feeder cells, OKT-3, and IL-2, to provide an expanded number of TILs.
31. The method of claim 30, wherein the tumor tissue in step (i) is trimmed to between 1.5 mm and 6 mm in diameter.
32. The method of claim 31, wherein the tumor tissue in step (i) is trimmed to about 6 mm by 6 mm by 6 mm.
33. The method of any one of claims 30 to 32, wherein the storage medium further comprises an antibacterial agent and an antifungal agent.
34. The method of any one of claims 30 to 33, wherein the storage medium comprises 2 % v/v to 12 % v/v dimethyl sulfoxide (DMSO).
35. The method of claim 34, wherein the storage medium comprises 10% DMSO.
36. The method of any one of claims 30 to 35, wherein the storage medium comprises gentamicin at a concentration of at least 50 µg/mL.
37. The method of any one of claims 30 to 36, wherein the tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing step (i).
38. The method of claim 37, wherein the washing comprises three serial washes of at least three minutes each, with the HBSS replaced after each serial wash.
39. The method of any one of claims 30 to 38, wherein step (b) comprises immersing the vessel in water bath at a temperature of about 37°C for about 5 minutes.
40. The method of claim 1 or claim 30, wherein the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal

tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, ovarian tumor tissue, or HPV-positive tumor tissue.

41. The method of claim 30, the method further comprising a split between the first expansion step (c) and the second expansion step (e).
42. The method of claim 41, wherein the split occurs about 16 days after step (c) is initiated.
43. The method of any one of claims 30 to 42, wherein the first culture step is about 11 days.
44. The method of any one of claims 30 to 42, wherein steps (c) through (e) are performed over a period of about 22 days.
45. The method of any one of claims 30 to 42, wherein steps (c) through (e) are performed over a period of less than 20 days.
46. The method of claim 30, wherein step (iv) comprises freezing the vessel at about -125 °C to about -150 °C.
47. The method of claim 30, wherein step (iv) comprises freezing the vessel using the vapor phase of liquid nitrogen.
48. The method of claim 30, wherein step (v) comprises storing the vessel below at least about -130 °C.
49. The method of claim 30, wherein step (v) comprises storing the vessel submerged in liquid nitrogen.
50. A method for preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy, the method comprising:
 - (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;

- (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at about 2 to about 8°C for about 30 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (b) thawing the vessel;
- (c) treating the tumor tissue in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 to provide TILs;
- (d) removing at least a plurality of the TILs;
- (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.
51. A method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:
- (a) storing the tumor tissue in a frozen state, the method of storing the tumor tissue comprising:
 - (i) trimming the tumor tissue to remove excess non-tumor tissue;
 - (ii) placing the tumor tissue in a closable vessel containing a storage medium;
 - (iii) incubating the vessel, which contains the tumor tissue and the storage medium, at 2 - 8°C for about 30 minutes;
 - (iv) freezing the vessel using the vapor phase of liquid nitrogen; and
 - (v) storing the vessel at the vapor phase of liquid nitrogen temperature;
 - (b) thawing the tumor tissue;
 - (c) adding the tumor fragments into a closed system;
 - (d) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first

expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (c) to step (d) occurs without opening the system;

- (e) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (d) to step (e) occurs without opening the system;
- (f) harvesting the therapeutic population of TILs obtained from step (e), wherein the transition from step (e) to step (f) occurs without opening the system;
- (g) transferring the harvested TIL population from step (f) to an infusion bag, wherein the transfer from step (f) to (g) occurs without opening the system;
- (h) cryopreserving the infusion bag comprising the harvested TIL population from step (g) using a cryopreservation process; and
- (i) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (h) to the subject.

52. The method of claim 51, further comprising the steps of (j) concomitantly administering a therapeutically effective dosage of aldesleukin or a biosimilar thereof to the subject and (k) concomitantly administering a therapeutically effective dosage of a PD-1/PD-L1 inhibitor to the subject.

53. The method of claim 52, wherein the PD-1/PD-L1 inhibitor is selected from the group consisting of pembrolizumab, nivolumab, avelumab, durvalumab, atezolizumab, and biosimilars thereof.

54. The method of claim 53, wherein the cancer is selected from the group consisting of melanoma, cervical cancer, head and neck squamous cell cancer, non-small cell lung cancer, bladder cancer, ovarian cancer, pancreatic cancer, and sarcoma.

55. A method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (e) obtaining tumor tissue from a patient;
 - (f) incubating the tumor tissue for at least 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C;
 - (g) freezing the tumor tissue;
 - (h) storing the tumor tissue in a frozen state;
 - (b) thawing the tumor tissue;
 - (c) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
 - (d) removing at least a plurality of the TILs; and
 - (e) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.
56. a method for expanding TILs from frozen tumor tissue comprises:
- (a) obtaining tumor tissue from a patient;
 - (b) trimming the tumor tissue to remove excess non-tumor tissue;
 - (b) placing the tumor tissue in a closable vessel containing a storage medium;

- (c) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
- (d) freezing the vessel;
- (e) storing the vessel such that the vessel remains frozen;
- (d) thawing the vessel;
- (f) treating the tumor tissue in a gas permeable container with a first cell culture medium and interleukin 2 (IL-2) to provide TILs;
- (g) removing at least a plurality of the TILs; and
- (h) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3, and IL-2 in a gas permeable container to provide an expanded number of TILs.

57. A method of treating cancer for a human subject in need thereof, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) produced by a method comprising:

- (a) obtaining tumor tissue from the subject;
- (b) trimming the tumor tissue to remove excess non-tumor tissue;
- (c) placing the tumor tissue in a closable vessel containing a storage medium;
- (d) incubating the vessel, which contains the tumor tissue and the storage medium, at a temperature in the range of about 2°C to about 8°C for about 20 minutes to about 70 minutes;
- (e) freezing the vessel using the vapor phase of liquid nitrogen;
- (f) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (g) thawing the tumor tissue;
- (h) adding the tumor tissue into a closed system;

- (i) performing a first expansion by culturing a first population of TILs from the tumor tissue in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (h) to step (i) occurs without opening the system;
- (j) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (i) to step (j) occurs without opening the system;
- (k) harvesting the therapeutic population of TILs obtained from step (j), wherein the transition from step (j) to step (k) occurs without opening the system;
- (l) transferring the therapeutic population of TILs harvested in step (k) to an infusion bag, wherein the transfer from step (k) to (l) occurs without opening the system;
- (m) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (l) using a cryopreservation process; and
- (n) administering a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (m) to the subject.

58. A method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) obtaining tumor tissue from a patient, wherein the tumor tissue comprises TILs;

- (b) incubating the tumor tissue for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C;
 - (c) freezing the tumor tissue;
 - (d) storing the tumor tissue in a frozen state;
 - (e) thawing the tumor tissue;
 - (f) treating the tumor tissue in a gas permeable container with a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;
 - (g) removing at least a plurality of the TILs; and
 - (h) expanding the TILs with a second cell culture medium comprising cell culture media, irradiated feeder cells, OKT-3 antibody, and IL-2 in a gas permeable container to provide an expanded number of TILs.
59. The method of claim 58, wherein the TILs in step (f) are cultured over a period of from about 3 to about 5 days, and wherein the TILs in step (h) are cultured over a period of from about 11 to about 13 days.
60. The method of claim 58, wherein the TILs in step (f) are cultured over a period of about 3 days, and wherein the TILs in step (h) are cultured over a period of about 13 days.
61. The method of claim 58, wherein the TILs in step (f) are cultured over a period of about 5 days, and wherein the TILs in step (h) are cultured over a period of about 11 days.
62. The method of claim 58, further comprising (i) harvesting the TILs.
63. The method of claim 61, wherein the harvested TILs represent at least a 10000-fold increase in number.
64. A method for preparing tumor infiltrating lymphocytes (TILs) for adoptive T-cell therapy, the method comprising:
- (a) culturing a cryopreserved tumor fragment in a first cell culture medium comprising interleukin 2 (IL-2) and optionally OKT-3 antibody to provide TILs;
 - (b) expanding the TILs in a second cell culture medium comprising irradiated feeder cells, OKT-3 antibody, and IL-2 to provide an expanded number of TILs; and,

- (c) optionally cryopreserving the expanded number of TILs.
65. The method of claim 64, wherein the first culture medium is a defined medium.
66. The method of claim 64, wherein the second culture medium is a defined medium.
67. The method of claim 64, wherein the first culture medium and the second culture medium are the same or different defined media.
68. The method of any of claims 64-67, wherein step (a) is performed in about 11 days.
69. The method of any of claims 64-67, wherein step (b) is performed in about 11 days.
70. The method of any of claims 64-68, wherein steps (a) and (b) are performed in a total of about 22 days.
71. The method of any of claims 64-67, wherein step (a) is performed in about 5 days.
72. The method of claim 71, wherein step (b) is performed in about 11 days.
73. The method of any of claims 64-67, wherein step (a) is performed in about 3 days.
74. The method of claim 73, wherein step (b) is performed in about 13 days.
75. The method of any of claims 64-67 or 71-74, wherein steps (a) and (b) are performed in a total of about 16 days.