

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

**[005]** In an example, the disclosure 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 example, the disclosure 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.

**[006a]** In one aspect, the present disclosure refers to 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.

**[007]** In an example, the disclosure 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.

**[007a]** In another aspect, the present disclosure refers to 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.

**[008]** In an example, the disclosure 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.

**[008a]** In another aspect, the present disclosure refers to 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.

**[008b]** In another aspect, the present disclosure refers to 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.

**[008c]** In another aspect, the present disclosure refers to 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.

**[008d]** In another aspect, the present disclosure refers to 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.

**[008e]** In another aspect, the present disclosure refers to 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.

**[009]** In an example, the disclosure 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 example, the disclosure 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.

**[0010a]** In another aspect, the present disclosure refers to a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) incubating a tumor tissue obtained from a patient for at least 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C;
- (b) freezing the tumor tissue;
- (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.

**[0011]** In some examples, the storage medium comprises 2 to 12 % v/v (volume:volume) dimethylsulfoxide (DMSO). In some examples the storage medium comprises 5% v/v DMSO. In some examples the storage medium comprises 10% v/v DMSO. In some examples, the storage medium comprises between 5 and 10 % v/v DMSO. In some examples 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 examples, the remainder of the storage medium is an aqueous medium.

**[0012]** In some examples, the storage medium comprises 2 to 12 % w/w (weight:weight) dimethylsulfoxide (DMSO). In some examples the storage medium comprises 5% w/w DMSO. In some examples the storage medium comprises 10% w/w DMSO. In some examples, the storage medium comprises between 5 and 10 % w/w DMSO. In some examples 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 examples, the remainder of the storage medium is an aqueous medium.

**[0013]** In some examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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 examples, 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.

**[0016a]** In another aspect, the present disclosure refers to a method for expanding TILs from frozen tumor tissue comprising:

- (a) trimming a tumor tissue obtained from a patient 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 examples, 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 examples, the fresh tumor tissue is trimmed to about 6 mm × 6 mm × 6 mm.

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

**[0019]** In some examples, 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 examples, 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 examples the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

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

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

**[0023]** In some examples, 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 examples, the method further comprises a split between the first expansion step (c) and the second expansion step (e). In an example, the split occurs on day 16. In some examples, the first culture step is complete in about 11 days. In other examples, the first expansion (or first culture) step is complete in about 7 days. In yet other examples, 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 examples, steps (c) through (e) are complete in about 22 days. In yet other examples, steps (c) through (e) are complete in 21 days; in some examples steps (c) through (e) are complete in about 20 days; and in some examples steps (c) through (e) are complete in about 16 days.

**[0025]** In some examples, the disclosure 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.

**[0025a]** In another aspect, the present disclosure refers to use of expanded tumor infiltrating lymphocytes (TILs) in the manufacture of a medicament for treating a subject with cancer, the use 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; and
- (h) cryopreserving the infusion bag comprising the harvested TIL population from step (g) using a cryopreservation process;
- (i) wherein a therapeutically effective dosage of the third population of TILs from the infusion bag in step (h) is to be administered to the subject.

**[0026]** In some examples, the disclosure 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.

**[0026a]** In another aspect, the present disclosure refers to use of expanded tumor infiltrating lymphocytes (TILs) in the manufacture of a medicament for treating cancer for a human subject in need thereof, wherein the expanded tumor infiltrating lymphocytes (TILs) are produced by a method comprising:

- (a) trimming a tumor tissue obtained from the subject 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 using the vapor phase of liquid nitrogen;
- (e) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (f) thawing the tumor tissue;
- (g) adding the tumor tissue into a closed system;
- (h) 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 (g) to step (h) occurs without opening the system;
- (i) 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 (h) to step (i) occurs without opening the system;
- (j) harvesting the therapeutic population of TILs obtained from step (i), wherein the transition from step (i) to step (j) occurs without opening the system;
- (k) transferring the therapeutic population of TILs harvested in step (j) to an infusion bag, wherein the transfer from step (j) to (k) occurs without opening the system; and
- (l) cryopreserving the infusion bag comprising the therapeutic population of TILs from step (k) using a cryopreservation process;
- (m) wherein a therapeutically effective dosage of the therapeutic population of TILs from the infusion bag in step (l) is to be administered to the subject.

**[0027]** In some examples, 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 disclosure 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.

**[0028a]** In another aspect, the present disclosure refers to a method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) incubating a tumor tissue obtained from a patient for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C, wherein the tumor tissue comprises TILs;
- (b) freezing the tumor tissue;
- (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 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.

**[0029]** The disclosure 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 examples, 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 examples, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing the freezing step of the method. In some examples, 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 examples the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

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

**[0034]** In some examples, 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 examples, the method further comprises a split between the first expansion (or first culture) step and the second expansion (or second culture) step. In an example, the split occurs on day 16. In some examples, the first culture step is complete in about 11 days. In other examples, the first expansion (or first culture) step is complete in about 7 days. In yet other examples, 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 examples, steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 22 days. In yet other examples, steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 21 days; in some examples steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 20 days; and in some examples steps (d) through (f) or steps (i) through (k), as applicable, are complete in about 16 days.

**[0036]** In some examples, the disclosure 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;

- (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.

**[0036a]** In another aspect, the present disclosure refers to 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.

**[0037]** In some examples, the fresh tumor tissue is first washed in Hank's Balanced Salt Solution (HBSS) before performing the freezing step of the method. In some examples, 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 examples the thawing step comprises immersing the vessel in a 37°C water bath for about 5 minutes.

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

**[0040]** In some examples, 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 examples, the method further comprises a split between the first expansion step and the second expansion step. In an example, the split occurs on day 16. In some examples, the first expansion step is complete in about 11 days. In other examples, the first expansion step is complete in about 7 days. In yet other examples, the first expansion

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

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

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

**[0044]** In some examples, 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 examples, 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 examples, feeder cells are used during the second expansion step. In an example, in a 1/100th scale process, about  $2\text{-}50 \times 10^6$  feeder cells are used during the second expansion step. In another example, about  $25 \times 10^6$  feeder cells are used. In another example, about  $50 \times 10^6$  feeder cells are used. In some examples, in a full scale process,

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

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

**[0047]** Other examples 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

## 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  $-130^{\circ}\text{C}$ .
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  $2^{\circ}\text{C}$  to about  $8^{\circ}\text{C}$  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  $2^{\circ}\text{C}$  to about  $8^{\circ}\text{C}$ ; 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. Use of expanded tumor infiltrating lymphocytes (TILs) in the manufacture of a medicament for treating a subject with cancer, the use 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; and
  - (h) cryopreserving the infusion bag comprising the harvested TIL population from step (g) using a cryopreservation process;
  - (i) wherein a therapeutically effective dosage of the third population of TILs from the infusion bag in step (h) is to be administered to the subject.

52. The use of claim 51, further comprising the steps of (j) wherein a therapeutically effective dosage of aldesleukin or a biosimilar thereof is to be administered to the subject and (k) wherein a therapeutically effective dosage of a PD-1/PD-L1 inhibitor is to be administered to the subject.

53. The use 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 use 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:

- (a) incubating a tumor tissue obtained from a patient for at least 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C;
- (b) freezing the tumor tissue;
- (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.

56. A method for expanding TILs from frozen tumor tissue comprising:

- (a) trimming a tumor tissue obtained from a patient 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. Use of expanded tumor infiltrating lymphocytes (TILs) in the manufacture of a medicament for treating cancer for a human subject in need thereof, wherein the expanded tumor infiltrating lymphocytes (TILs) are produced by a method comprising:

- (a) trimming a tumor tissue obtained from the subject 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 using the vapor phase of liquid nitrogen;
- (e) storing the vessel at the vapor phase of liquid nitrogen temperature;
- (f) thawing the tumor tissue;
- (g) adding the tumor tissue into a closed system;
- (h) 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 (g) to step (h) occurs without opening the system;

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

58. A method for expanding tumor infiltrating lymphocytes (TILs) from frozen tumor tissue comprising:

- (a) incubating a tumor tissue obtained from a patient for about 30 minutes in a storage medium at a temperature in the range of about 2°C to about 8°C, wherein the tumor tissue comprises TILs;
- (b) freezing the tumor tissue;
- (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 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.
59. The method of claim 58, wherein the TILs in step (e) are cultured over a period of from about 3 to about 5 days, and wherein the TILs in step (g) are cultured over a period of from about 11 to about 13 days.
60. The method of claim 58, wherein the TILs in step (e) are cultured over a period of about 3 days, and wherein the TILs in step (g) are cultured over a period of about 13 days.
61. The method of claim 58, wherein the TILs in step (e) are cultured over a period of about 5 days, and wherein the TILs in step (g) are cultured over a period of about 11 days.
62. The method of claim 58, further comprising (h) 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.