

TWO-DIMENSIONAL PROCESSES FOR THE EXPANSION OF TUMOR INFILTRATING LYMPHOCYTES AND THERAPIES THEREFROM

CROSS-REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to U.S. provisional application No. 63/384,576 filed November 21, 2022, the entire disclosure of which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Treatment of bulky, refractory cancers using adoptive autologous transfer of tumor infiltrating lymphocytes (TILs) represents a powerful approach to therapy for patients with poor prognoses. Gattinoni, *et al.*, *Nat. Rev. Immunol.* **2006**, *6*, 383-393. TILs are dominated by T cells, and 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. A number of approaches to improve responses to TIL therapy in melanoma and to expand TIL therapy to other tumor types have been explored with limited success, and the field remains challenging. Goff, *et al.*, *J. Clin. Oncol.* **2016**, *34*, 2389-97; Dudley, *et al.*, *J. Clin. Oncol.* **2008**, *26*, 5233-39; Rosenberg, *et al.*, *Clin. Cancer Res.* **2011**, *17*, 4550-57. Combination studies with single immune checkpoint inhibitors have also been described, but further studies are ongoing and additional methods of treatment are needed (Kverneland, *et al.*, *Oncotarget*, **2020**, *11*(22), 2092-2105).

[0003] TILs naturally infiltrate tumors; however, reports suggest that large numbers of these are “bystander TILs” that are not reactive to the tumor-specific neoantigens. Gokuldass, A., *et al. Cancers*, **2020**, *12*(11), 3344. As a result of this imbalanced, non-targeted expansion regimens may result in disproportionate amounts of bystander TILs in a therapeutic population. There remains a need in the art for methods of identifying tumor-reactive TILs within a population of TILs. and to specifically expand this tumor-reactive subpopulation. The present disclosure meets this need by providing methods of identification and expansion of tumor-reactive TIL subpopulations and compositions derived therefrom.

BRIEF SUMMARY OF THE INVENTION

[0004] Provided herein are methods for enriching and identifying tumor-reactive cells within a sample of tumor such that they are distinguished from bystander TILs. In one aspect, the disclosure herein further comprises methods for expansion of previously-identified tumor-reactive TILs.

[0005] In some embodiments, the method comprises the steps of:

- (a) obtaining a tumor sample from a patient;
- (b) dividing the tumor sample into a first portion and a second portion;
- (c) performing a first expansion of a first population of TILs in the second portion of the tumor sample by culturing the second portion of the tumor sample in a first cell culture medium and IL-2 to produce a second population of TILs; and
- (d) contacting the second population of TILs with tumor cells or tumor cell antigens from or derived from a tumor digest obtained by digesting the first portion of the tumor sample to generate a third population of TILs,
wherein the third population of TILs comprises a plurality of tumor reactive TILs that is enriched in comparison to the second population of TILs.

[0006] In some embodiments, step (d) is performed for about 1 to about 3 days.

[0007] In some embodiments, the method comprises the steps of:

- (a) obtaining a tumor sample from a patient;
- (b) dividing the tumor sample into a first portion and a second portion;
- (c) performing a first expansion of a first population of TILs in the second portion of the tumor sample by culturing the second portion of the tumor sample in a first cell culture medium and IL-2 to produce a second population of TILs; and
- (d) contacting the second population of TILs with a population of mature dendritic cells (DCs) generated from culturing a population of immature DCs with tumor cells or tumor cell antigens from or derived from a tumor digest obtained by digesting the first portion

of the tumor sample to generate a third population of TILs, wherein the third population of TILs comprises a plurality of tumor reactive TILs that is enriched in comparison to the second population of TILs.

[0008] In some embodiments, the population of immature DCs is generated by culturing a population of monocytes in the presence of GM-CSF and IL-4. In some embodiments, the population of monocytes is obtained from PBMCs. In some embodiments, the PBMCs are obtained from the patient. In some embodiments, the population of monocytes is cultured in the presence of GM-CSF and IL-4 for about 6 days. In some embodiments, culturing the population of immature DCs in the presence of tumor cells or tumor cell antigens from or derived from the tumor digest comprises generating tumor lysate from the tumor digest and culturing the immature DCs in the presence of tumor cells or tumor cell antigens from or derived from the tumor lysate. In some embodiments, the population of immature DCs is cultured with the tumor cells of the tumor digest at a ratio of about 3:1. In some embodiments, the population of immature DCs is cultured in the presence of tumor cells or tumor cell antigens from or derived from the tumor digest for about 24 hours. In some embodiments, the tumor digest or tumor lysate is subjected to dead cell removal prior to being cultured with the population of immature DCs. In some embodiments, culturing the population of immature DCs in the presence of tumor cells or tumor cell antigens from or derived from the tumor digest is performed in the presence of TNF α , IL-6 and IL-1 β . In some embodiments, the concentration of TNF α is about 2000IU/ml. In some embodiments, the concentration of IL-6 is about 2000IU/ml. In some embodiments, the concentration of IL-1 β is about 400IU/ml. In some embodiments, the second population of TILs are cultured with the mature DCs. In some embodiments, step (d) is performed for about 1-3 days.

[0009] In some embodiments, the method comprises the steps of:

- (a) obtaining a tumor sample from a patient;
- (b) dividing the tumor sample into a first portion and a second portion;
- (c) performing a first expansion of a first population of TILs in the second portion of the tumor sample by culturing the second portion of the tumor sample in a first cell culture medium and IL-2 to produce a second population of TILs; and

- (d) contacting the second population of TILs with organoids generated from the first portion of the tumor sample to generate a third population of TILs, wherein the third population of TILs comprises a plurality of tumor reactive TILs that is enriched in comparison to the second population of TILs.

[0010] In some embodiments, generating organoids from the first portion of the tumor sample comprises digesting the first portion of the tumor sample to obtain a tumor digest and generating organoids from the tumor digest.

[0011] In some embodiments, generating organoids comprises:

- (a) driving the first portion of the tumor sample and an unpolymerized fluid matrix material through one or more channels of a microfluidics apparatus,
 - i. wherein the microfluidics apparatus controls the pressure, flow rate or pressure and flow rate within the one or more channels and maintains a temperature of 20 degrees C or less, so that tumor-derived cells or multiple tumor fragments in the tumor sample and the unpolymerized fluid matrix travel through the one or more channels in laminar flow,
- (b) combining tumor-derived cells or multiple tumor fragments and the unpolymerized fluid matrix material within the microfluidics apparatus to form a plurality of droplets of unpolymerized mixture, and
- (c) exposing the plurality of droplets of unpolymerized mixture to a temperature of greater than 25 degrees C to polymerize the fluid matrix material and form the organoid.

[0012] In some embodiments, the methods disclosed herein further comprise identifying the plurality of tumor reactive TILs in the third population of TILs. In some embodiments, identifying the plurality of tumor reactive TILs comprises determining if a TIL exhibits an activation signal identifying the TIL as tumor reactive. In some embodiments, the activation signal comprises increased and/or decreased cell surface expression of one or more proteins. In some embodiments, the one or more proteins are selected from the group consisting of: CD3, CD4, CD8, PD-1, LAG3, Tim3, TIGIT, CD103, CD39, CD134, CD137, CD25, CD69, HLA-DR, CD107a, CD40L, Ki46, CD45RA, CCR7, and KLRG1. In some embodiments, the cell surface expression of the one or more proteins is determined by flow cytometry. In some

embodiments, the flow cytometry is performed using a SONY FX 500, Miltenyi Tyto or Miltenyi CliniMACS flow-activated cell sorter. In some embodiments, the activation signal comprises a cell morphology. In some embodiments, the cell morphology is a flattened, rounded cell morphology. In some embodiments, the activation signal is a concentration of mitochondrial mass in proximity to the cell membrane of the TIL. In some embodiments, the activation signal is determined by an imaging-based cell separation method.

[0013] In some embodiments, the methods disclosed herein further comprise collecting the identified plurality of tumor reactive TILs. In some embodiments, collecting the plurality of tumor reactive TILs comprises separating the plurality of tumor reactive TILs from non-tumor reactive TILs in the third population of TILs. In some embodiments, the separating the plurality of tumor reactive TILs comprises removing non-tumor reactive TILs from the third population of TILs.

[0014] In some embodiments, the methods disclosed herein further comprise performing the step of:

- (e) performing a second expansion by culturing the third population of TILs or the collected plurality of tumor reactive TILs in a second cell culture medium supplemented with additional IL-2, OKT-3 and antigen-presenting cells to generate a fourth population of TILs.

[0015] In some embodiments, the first portion of the tumor sample comprises approximately one-third of the tumor sample. In some embodiments, the second portion of the tumor sample comprises approximately one half of the tumor sample. In some embodiments, the second portion of the tumor sample comprises approximately one third of the tumor sample. In some embodiments, the tumor digest undergoes 1, 2, 3, 4, 5, or 10 freeze-thaw cycles. In some embodiments, the first portion of the tumor sample comprises at least three million cells. In some embodiments, steps (a) through (e) are performed within a period of about 17 days to about 24 days, within a period of about 18 days to about 22 days, within a period of about 20 days to about 22 days, or within a period of about 22 days. In some embodiments, the first cell culture medium further comprises a factor selected from the group consisting of: IL-7, IL-15, IL-21, IL-12, Leukemia Inhibitory Factor (LIF), beta fibroblast growth factor (bFGF), and combinations thereof. In some embodiments, the second cell culture medium further comprises a factor

selected from the group consisting of: IL-7, IL-15, IL-21, IL-12, LIF, bFGF, 41BBL, OX40L, CD86, CD64, and combinations thereof. In some embodiments, the tumor sample is selected from the group consisting of a solid tumor, a fine needle aspirate, and a small biopsy. In some embodiments, the activation signal comprises an increase in secreted interferon gamma (IFN γ). In some embodiments, the cell culture medium is provided in a container selected from the group consisting of a G-container and a Xuri cellbag.

[0016] In some embodiments, the methods disclosed herein further comprise gene-editing the second population of TILs, the third population of TILs, or the plurality of tumor reactive TILs.

[0017] Further provided herein is a pharmaceutical composition for the treatment of cancer comprising the fourth population of TILs generated using the methods disclosed herein.

[0018] In some embodiments, the cancer is selected for the group consisting of melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, triple negative 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 pharmaceutical composition further comprises a cryopreservant. In some embodiments, the cryopreservant comprises dimethylsulfoxide. In some embodiments, the pharmaceutical composition further comprises a cryopreservant and an isotonic agent. In some embodiments, the pharmaceutical composition further comprises a cryopreservant comprising dimethylsulfoxide and an isotonic agent comprising sodium chloride, sodium gluconate, and sodium acetate. In some embodiments, the pharmaceutical composition further comprises a cryopreservant comprising dimethylsulfoxide and dextran 40 and an isotonic agent comprising sodium chloride, sodium gluconate, and sodium acetate. In some embodiments, the composition is provided in a sterile infusion bag. In some embodiments, said composition is harvested using a LOVO cell processing system. In some embodiments, the TILs are gene-edited.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] **Figure 1** illustrates an embodiment of the processes for enriching tumor reactive TILs using autologous tumor digest.

[0020] **Figure 2** illustrates an embodiment of the processes for enriching tumor reactive TILs using autologous dendritic cells (DCs) or DC like cells.

[0021] **Figure 3** illustrates an embodiment of the processes for enriching tumor reactive TILs using autologous organoids/tumoroids.

[0022] **Figures 4A & 4B** show the results from TIL:tumor cell co-cultures.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

[0023] SEQ ID NO:1 is the amino acid sequence of the heavy chain of muromonab.

[0024] SEQ ID NO:2 is the amino acid sequence of the light chain of muromonab.

[0025] SEQ ID NO:3 is the amino acid sequence of a recombinant human IL-2 protein.

[0026] SEQ ID NO:4 is the amino acid sequence of aldesleukin.

[0027] SEQ ID NO:5 is an IL-2 form.

[0028] SEQ ID NO:6 is the amino acid sequence of nemvaleukin alfa.

[0029] SEQ ID NO:7 is an IL-2 form.

[0030] SEQ ID NO:8 is a mucin domain polypeptide.

[0031] SEQ ID NO:9 is the amino acid sequence of a recombinant human IL-4 protein.

[0032] SEQ ID NO:10 is the amino acid sequence of a recombinant human IL-7 protein.

[0033] SEQ ID NO:11 is the amino acid sequence of a recombinant human IL-15 protein.

[0034] SEQ ID NO:12 is the amino acid sequence of a recombinant human IL-21 protein.

[0035] SEQ ID NO:13 is an IL-2 sequence.

[0036] SEQ ID NO:14 is an IL-2 mutein sequence.

[0037] SEQ ID NO:15 is an IL-2 mutein sequence.

[0038] SEQ ID NO:16 is the HCDR1_IL-2 for IgG.IL2R67A.H1.

[0039] SEQ ID NO:17 is the HCDR2 for IgG.IL2R67A.H1.

[0040] SEQ ID NO:18 is the HCDR3 for IgG.IL2R67A.H1.

[0041] SEQ ID NO:19 is the HCDR1_IL-2 kabat for IgG.IL2R67A.H1.

- [0042] SEQ ID NO:20 is the HCDR2 kabat for IgG.IL2R67A.H1.
- [0043] SEQ ID NO:21 is the HCDR3 kabat for IgG.IL2R67A.H1.
- [0044] SEQ ID NO:22 is the HCDR1_IL-2 clothia for IgG.IL2R67A.H1.
- [0045] SEQ ID NO:23 is the HCDR2 clothia for IgG.IL2R67A.H1.
- [0046] SEQ ID NO:24 is the HCDR3 clothia for IgG.IL2R67A.H1.
- [0047] SEQ ID NO:25 is the HCDR1_IL-2 IMGT for IgG.IL2R67A.H1.
- [0048] SEQ ID NO:26 is the HCDR2 IMGT for IgG.IL2R67A.H1.
- [0049] SEQ ID NO:27 is the HCDR3 IMGT for IgG.IL2R67A.H1.
- [0050] SEQ ID NO:28 is the VH chain for IgG.IL2R67A.H1.
- [0051] SEQ ID NO:29 is the heavy chain for IgG.IL2R67A.H1.
- [0052] SEQ ID NO:30 is the LCDR1 kabat for IgG.IL2R67A.H1.
- [0053] SEQ ID NO:31 is the LCDR2 kabat for IgG.IL2R67A.H1.
- [0054] SEQ ID NO:32 is the LCDR3 kabat for IgG.IL2R67A.H1.
- [0055] SEQ ID NO:33 is the LCDR1 chothia for IgG.IL2R67A.H1.
- [0056] SEQ ID NO:34 is the LCDR2 chothia for IgG.IL2R67A.H1.
- [0057] SEQ ID NO:35 is the LCDR3 chothia for IgG.IL2R67A.H1.
- [0058] SEQ ID NO:36 is a VL chain.
- [0059] SEQ ID NO:37 is a light chain.
- [0060] SEQ ID NO:38 is a light chain.
- [0061] SEQ ID NO:39 is a light chain.
- [0062] SEQ ID NO:40 is the amino acid sequence of human 4-1BB.
- [0063] SEQ ID NO:41 is the amino acid sequence of murine 4-1BB.
- [0064] SEQ ID NO:42 is the heavy chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0065] SEQ ID NO:43 is the light chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0066] SEQ ID NO:44 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0067] SEQ ID NO:45 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0068] SEQ ID NO:46 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0069] SEQ ID NO:47 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0070] SEQ ID NO:48 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0071] SEQ ID NO:49 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0072] SEQ ID NO:50 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0073] SEQ ID NO:51 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0074] SEQ ID NO:52 is the heavy chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0075] SEQ ID NO:53 is the light chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0076] SEQ ID NO:54 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0077] SEQ ID NO:55 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0078] SEQ ID NO:56 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0079] SEQ ID NO:57 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0080] SEQ ID NO:58 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0081] SEQ ID NO:59 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0082] SEQ ID NO:60 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0083] SEQ ID NO:61 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0084] SEQ ID NO:62 is an Fc domain for a TNFRSF agonist fusion protein.

[0085] SEQ ID NO:63 is a linker for a TNFRSF agonist fusion protein.

[0086] SEQ ID NO:64 is a linker for a TNFRSF agonist fusion protein.

[0087] SEQ ID NO:65 is a linker for a TNFRSF agonist fusion protein.

[0088] SEQ ID NO:66 is a linker for a TNFRSF agonist fusion protein.

[0089] SEQ ID NO:67 is a linker for a TNFRSF agonist fusion protein.

[0090] SEQ ID NO:68 is a linker for a TNFRSF agonist fusion protein.

[0091] SEQ ID NO:69 is a linker for a TNFRSF agonist fusion protein.

[0092] SEQ ID NO:70 is a linker for a TNFRSF agonist fusion protein.

[0093] SEQ ID NO:71 is a linker for a TNFRSF agonist fusion protein.

[0094] SEQ ID NO:72 is a linker for a TNFRSF agonist fusion protein.

[0095] SEQ ID NO:73 is an Fc domain for a TNFRSF agonist fusion protein.

[0096] SEQ ID NO:74 is a linker for a TNFRSF agonist fusion protein.

[0097] SEQ ID NO:75 is a linker for a TNFRSF agonist fusion protein.

- [0098] SEQ ID NO:76 is a linker for a TNFRSF agonist fusion protein.
- [0099] SEQ ID NO:77 is a 4-1BB ligand (4-1BBL) amino acid sequence.
- [0100] SEQ ID NO:78 is a soluble portion of 4-1BBL polypeptide.
- [0101] SEQ ID NO:79 is a heavy chain variable region (VH) for the 4-1BB agonist antibody 4B4-1-1 version 1.
- [0102] SEQ ID NO:80 is a light chain variable region (VL) for the 4-1BB agonist antibody 4B4-1-1 version 1.
- [0103] SEQ ID NO:81 is a heavy chain variable region (VH) for the 4-1BB agonist antibody 4B4-1-1 version 2.
- [0104] SEQ ID NO:82 is a light chain variable region (VL) for the 4-1BB agonist antibody 4B4-1-1 version 2.
- [0105] SEQ ID NO:83 is a heavy chain variable region (VH) for the 4-1BB agonist antibody H39E3-2.
- [0106] SEQ ID NO:84 is a light chain variable region (VL) for the 4-1BB agonist antibody H39E3-2.
- [0107] SEQ ID NO:85 is the amino acid sequence of human OX40.
- [0108] SEQ ID NO:86 is the amino acid sequence of murine OX40.
- [0109] SEQ ID NO:87 is the heavy chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [0110] SEQ ID NO:88 is the light chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [0111] SEQ ID NO:89 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [0112] SEQ ID NO:90 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [0113] SEQ ID NO:91 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

- [00114] SEQ ID NO:92 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [00115] SEQ ID NO:93 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [00116] SEQ ID NO:94 is the light chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [00117] SEQ ID NO:95 is the light chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [00118] SEQ ID NO:96 is the light chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).
- [00119] SEQ ID NO:97 is the heavy chain for the OX40 agonist monoclonal antibody 11D4.
- [00120] SEQ ID NO:98 is the light chain for the OX40 agonist monoclonal antibody 11D4.
- [00121] SEQ ID NO:99 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 11D4.
- [00122] SEQ ID NO:100 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 11D4.
- [00123] SEQ ID NO:101 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody 11D4.
- [00124] SEQ ID NO:102 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 11D4.
- [00125] SEQ ID NO:103 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 11D4.
- [00126] SEQ ID NO:104 is the light chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

- [00127] SEQ ID NO:105 is the light chain CDR2 for the OX40 agonist monoclonal antibody 11D4.
- [00128] SEQ ID NO:106 is the light chain CDR3 for the OX40 agonist monoclonal antibody 11D4.
- [00129] SEQ ID NO:107 is the heavy chain for the OX40 agonist monoclonal antibody 18D8.
- [00130] SEQ ID NO:108 is the light chain for the OX40 agonist monoclonal antibody 18D8.
- [00131] SEQ ID NO:109 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 18D8.
- [00132] SEQ ID NO:110 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 18D8.
- [00133] SEQ ID NO:111 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody 18D8.
- [00134] SEQ ID NO:112 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 18D8.
- [00135] SEQ ID NO:113 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 18D8.
- [00136] SEQ ID NO:114 is the light chain CDR1 for the OX40 agonist monoclonal antibody 18D8.
- [00137] SEQ ID NO:115 is the light chain CDR2 for the OX40 agonist monoclonal antibody 18D8.
- [00138] SEQ ID NO:116 is the light chain CDR3 for the OX40 agonist monoclonal antibody 18D8.
- [00139] SEQ ID NO:117 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody Hu119-122.

- [00140] SEQ ID NO:118 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody Hu119-122.
- [00141] SEQ ID NO:119 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.
- [00142] SEQ ID NO:120 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.
- [00143] SEQ ID NO:121 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.
- [00144] SEQ ID NO:122 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.
- [00145] SEQ ID NO:123 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.
- [00146] SEQ ID NO:124 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.
- [00147] SEQ ID NO:125 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody Hu106-222.
- [00148] SEQ ID NO:126 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody Hu106-222.
- [00149] SEQ ID NO:127 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.
- [00150] SEQ ID NO:128 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.
- [00151] SEQ ID NO:129 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.
- [00152] SEQ ID NO:130 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.

- [00153] SEQ ID NO:131 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.
- [00154] SEQ ID NO:132 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.
- [00155] SEQ ID NO:133 is an OX40 ligand (OX40L) amino acid sequence.
- [00156] SEQ ID NO:134 is a soluble portion of OX40L polypeptide.
- [00157] SEQ ID NO:135 is an alternative soluble portion of OX40L polypeptide.
- [00158] SEQ ID NO:136 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 008.
- [00159] SEQ ID NO:137 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 008.
- [00160] SEQ ID NO:138 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 011.
- [00161] SEQ ID NO:139 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 011.
- [00162] SEQ ID NO:140 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 021.
- [00163] SEQ ID NO:141 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 021.
- [00164] SEQ ID NO:142 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 023.
- [00165] SEQ ID NO:143 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 023.
- [00166] SEQ ID NO:144 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.
- [00167] SEQ ID NO:145 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

- [00168] SEQ ID NO:146 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.
- [00169] SEQ ID NO:147 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.
- [00170] SEQ ID NO:148 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.
- [00171] SEQ ID NO:149 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.
- [00172] SEQ ID NO:150 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.
- [00173] SEQ ID NO:151 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.
- [00174] SEQ ID NO:152 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.
- [00175] SEQ ID NO:153 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.
- [00176] SEQ ID NO:154 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.
- [00177] SEQ ID NO:155 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.
- [00178] SEQ ID NO:156 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.
- [00179] SEQ ID NO:157 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.
- [00180] SEQ ID NO:158 is the heavy chain amino acid sequence of the PD-1 inhibitor nivolumab.

- [00181] SEQ ID NO:159 is the light chain amino acid sequence of the PD-1 inhibitor nivolumab.
- [00182] SEQ ID NO:160 is the heavy chain variable region (VH) amino acid sequence of the PD-1 inhibitor nivolumab.
- [00183] SEQ ID NO:161 is the light chain variable region (VL) amino acid sequence of the PD-1 inhibitor nivolumab.
- [00184] SEQ ID NO:162 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.
- [00185] SEQ ID NO:163 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.
- [00186] SEQ ID NO:164 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.
- [00187] SEQ ID NO:165 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.
- [00188] SEQ ID NO:166 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.
- [00189] SEQ ID NO:167 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.
- [00190] SEQ ID NO:168 is the heavy chain amino acid sequence of the PD-1 inhibitor pembrolizumab.
- [00191] SEQ ID NO:169 is the light chain amino acid sequence of the PD-1 inhibitor pembrolizumab.
- [00192] SEQ ID NO:170 is the heavy chain variable region (VH) amino acid sequence of the PD-1 inhibitor pembrolizumab.
- [00193] SEQ ID NO:171 is the light chain variable region (VL) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00194] SEQ ID NO:172 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00195] SEQ ID NO:173 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00196] SEQ ID NO:174 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00197] SEQ ID NO:175 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00198] SEQ ID NO:176 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00199] SEQ ID NO:177 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00200] SEQ ID NO:178 is the heavy chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00201] SEQ ID NO:179 is the light chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00202] SEQ ID NO:180 is the heavy chain variable region (VH) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00203] SEQ ID NO:181 is the light chain variable region (VL) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00204] SEQ ID NO:182 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00205] SEQ ID NO:183 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00206] SEQ ID NO:184 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

- [00207] SEQ ID NO:185 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.
- [00208] SEQ ID NO:186 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.
- [00209] SEQ ID NO:187 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.
- [00210] SEQ ID NO:188 is the heavy chain amino acid sequence of the PD-L1 inhibitor avelumab.
- [00211] SEQ ID NO:189 is the light chain amino acid sequence of the PD-L1 inhibitor avelumab.
- [00212] SEQ ID NO:190 is the heavy chain variable region (VH) amino acid sequence of the PD-L1 inhibitor avelumab.
- [00213] SEQ ID NO:191 is the light chain variable region (VL) amino acid sequence of the PD-L1 inhibitor avelumab.
- [00214] SEQ ID NO:192 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.
- [00215] SEQ ID NO:193 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.
- [00216] SEQ ID NO:194 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.
- [00217] SEQ ID NO:195 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.
- [00218] SEQ ID NO:196 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.
- [00219] SEQ ID NO:197 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

- [00220] SEQ ID NO:198 is the heavy chain amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00221] SEQ ID NO:199 is the light chain amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00222] SEQ ID NO:200 is the heavy chain variable region (VH) amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00223] SEQ ID NO:201 is the light chain variable region (VL) amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00224] SEQ ID NO:202 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00225] SEQ ID NO:203 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00226] SEQ ID NO:204 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00227] SEQ ID NO:205 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00228] SEQ ID NO:206 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00229] SEQ ID NO:207 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.
- [00230] SEQ ID NO:208 is the heavy chain amino acid sequence of the CTLA-4 inhibitor ipilimumab.
- [00231] SEQ ID NO:209 is the light chain amino acid sequence of the CTLA-4 inhibitor ipilimumab.
- [00232] SEQ ID NO:210 is the heavy chain variable region (VH) amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00233] SEQ ID NO:211 is the light chain variable region (VL) amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00234] SEQ ID NO:212 is the heavy chain CDR1 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00235] SEQ ID NO:213 is the heavy chain CDR2 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00236] SEQ ID NO:214 is the heavy chain CDR3 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00237] SEQ ID NO:215 is the light chain CDR1 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00238] SEQ ID NO:216 is the light chain CDR2 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00239] SEQ ID NO:217 is the light chain CDR3 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00240] SEQ ID NO:218 is the heavy chain amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00241] SEQ ID NO:219 is the light chain amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00242] SEQ ID NO:220 is the heavy chain variable region (VH) amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00243] SEQ ID NO:221 is the light chain variable region (VL) amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00244] SEQ ID NO:222 is the heavy chain CDR1 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00245] SEQ ID NO:223 is the heavy chain CDR2 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00246] SEQ ID NO:224 is the heavy chain CDR3 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00247] SEQ ID NO:225 is the light chain CDR1 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00248] SEQ ID NO:226 is the light chain CDR2 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00249] SEQ ID NO:227 is the light chain CDR3 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00250] SEQ ID NO:228 is the heavy chain amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00251] SEQ ID NO:229 is the light chain amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00252] SEQ ID NO:230 is the heavy chain variable region (VH) amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00253] SEQ ID NO:231 is the light chain variable region (VL) amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00254] SEQ ID NO:232 is the heavy chain CDR1 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00255] SEQ ID NO:233 is the heavy chain CDR2 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00256] SEQ ID NO:234 is the heavy chain CDR3 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00257] SEQ ID NO:235 is the light chain CDR1 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00258] SEQ ID NO:236 is the light chain CDR2 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00259] SEQ ID NO:237 is the light chain CDR3 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

I. Definitions

[00260] 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.

[00261] The terms “co-administration,” “co-administering,” “administered in combination with,” “administering in combination with,” “simultaneous,” and “concurrent,” as used herein, encompass administration of two or more active pharmaceutical ingredients (in a preferred embodiment of the present invention, for example, a plurality of TILs) to a subject so that both active pharmaceutical ingredients and/or their metabolites are present in the subject at the same time. Co-administration includes simultaneous administration in separate compositions, administration at different times in separate compositions, or administration in a composition in which two or more active pharmaceutical ingredients are present. Simultaneous administration in separate compositions and administration in a composition in which both agents are present are preferred.

[00262] The term “*in vivo*” refers to an event that takes place in a subject's body.

[00263] 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.

[00264] 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.

[00265] The term “rapid expansion” means an increase in the number of antigen-specific TILs of at least about 3-fold (or 4-, 5-, 6-, 7-, 8-, or 9-fold) over a period of a week, more preferably at least about 10-fold (or 20-, 30-, 40-, 50-, 60-, 70-, 80-, or 90-fold) over a period of a week, or most preferably at least about 100-fold over a period of a week. A number of rapid expansion protocols are described herein.

[00266] 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.

[00267] 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.

[00268] By “cryopreserved TILs” herein is meant that TILs, either primary, bulk, or expanded (REP TILs), are treated and stored in the range of about -150°C to -60°C. General methods for cryopreservation are also described elsewhere herein, including in the Examples. For clarity, “cryopreserved TILs” are distinguishable from frozen tissue samples which may be used as a source of primary TILs.

[00269] 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.

[00270] 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.

[00271] The term “cryopreservation media” or “cryopreservation medium” refers to any medium that can be used for cryopreservation of cells. Such media can include media comprising 7% to 10% DMSO. Exemplary media include CryoStor CS10, Hyperthermasol, as well as combinations thereof. The term “CS10” refers to a cryopreservation medium which is obtained from Stemcell Technologies or from Biolife Solutions. The CS10 medium may be referred to by the trade name “CryoStor® CS10”. The CS10 medium is a serum-free, animal component-free medium which comprises DMSO. In some embodiments, the CS10 medium comprises 10% DMSO.

[00272] 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.

[00273] 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.

[00274] The term “closed system” refers to a system that is closed to the outside environment. Any closed system appropriate for cell culture methods can be employed with the methods of the present invention. Closed systems include, for example, but are not limited to, closed G-containers. Once a tumor segment is added to the closed system, the system is no opened to the outside environment until the TILs are ready to be administered to the patient.

[00275] 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.

[00276] The terms “peripheral blood mononuclear cells” and “PBMCs” refers to a peripheral blood cell having a round nucleus, including lymphocytes (T cells, B cells, NK cells) and monocytes. When used as an antigen presenting cell (PBMCs are a type of antigen-presenting cell), the peripheral blood mononuclear cells are preferably irradiated allogeneic peripheral blood mononuclear cells.

[00277] The terms “peripheral blood lymphocytes” and “PBLs” refer to T cells expanded from peripheral blood. In some embodiments, PBLs are separated from whole blood or apheresis product from a donor. In some embodiments, PBLs are separated from whole blood or apheresis product from a donor by positive or negative selection of a T cell phenotype, such as the T cell phenotype of CD3⁺ CD45⁺.

[00278] The term “anti-CD3 antibody” refers to an antibody or variant thereof, *e.g.*, a monoclonal antibody and including human, humanized, chimeric or murine antibodies which are directed against the CD3 receptor in the T cell antigen receptor of mature T cells. Anti-CD3 antibodies include OKT-3, also known as muromonab. Anti-CD3 antibodies also include the UHCT1 clone, also known as T3 and CD3ε. Other anti-CD3 antibodies include, for example, oteixizumab, teplizumab, and visilizumab.

[00279] 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 (exemplary OKT-3 antibody).

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:1 muromonab heavy chain	QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY 60 NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTTLTVSSA 120 KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFEPVTLTW NSGSLSSGVH TFAVLQSDL 180 YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG 240 PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN 300 STYRVVSVLT VLNQDNLNGK 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 MTCASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH 60 FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS 120 SEQLTSGGAS VVCFLLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL 180 TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC 213

[00280] 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 bempegaldesleukin (NKTR-214, pegylated human recombinant IL-2 as in SEQ ID NO:4 in which an average of 6 lysine residues are N⁶ substituted with [(2,7-bis{[methylpoly(oxyethylene)]carbamoyl}-9H-fluoren-9-yl)methoxy]carbonyl), which is available from Nektar Therapeutics, South San Francisco, CA, USA, or which may be prepared by methods known in the art, such as the methods described in Example 19 of International

Patent Application Publication No. WO 2018/132496 A1 or the method described in Example 1 of U.S. Patent Application Publication No. US 2019/0275133 A1, the disclosures of which are incorporated by reference herein. Bempegaldesleukin (NKTR-214) and other pegylated IL-2 molecules suitable for use in the invention are 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.

[0001] In some embodiments, an IL-2 form suitable for use in the present invention is THOR-707, available from Synthorx, Inc. The preparation and properties of THOR-707 and additional alternative forms of IL-2 suitable for use in the invention are described in U.S. Patent Application Publication Nos. US 2020/0181220 A1 and US 2020/0330601 A1, the disclosures of which are incorporated by reference herein. In some embodiments, an IL-2 form suitable for use in the invention is an interleukin 2 (IL-2) conjugate comprising: an isolated and purified IL-2 polypeptide; and a conjugating moiety that binds to the isolated and purified IL-2 polypeptide at an amino acid position selected from K35, T37, R38, T41, F42, K43, F44, Y45, E61, E62, E68, K64, P65, V69, L72, and Y107, wherein the numbering of the amino acid residues corresponds to SEQ ID NO:5. In some embodiments, the amino acid position is selected from T37, R38, T41, F42, F44, Y45, E61, E62, E68, K64, P65, V69, L72, and Y107. In some embodiments, the amino acid position is selected from T37, R38, T41, F42, F44, Y45, E61, E62, E68, P65, V69, L72, and Y107. In some embodiments, the amino acid position is selected from T37, T41, F42, F44, Y45, P65, V69, L72, and Y107. In some embodiments, the amino acid position is selected from R38 and K64. In some embodiments, the amino acid position is selected from E61, E62, and E68. In some embodiments, the amino acid position is at E62. In some embodiments, the amino acid residue selected from K35, T37, R38, T41, F42, K43, F44, Y45, E61, E62, E68, K64, P65, V69, L72, and Y107 is further mutated to lysine, cysteine, or histidine. In some embodiments, the amino acid residue is mutated to cysteine. In some embodiments, the amino acid residue is mutated to lysine. In some embodiments, the amino acid residue selected from K35, T37, R38, T41, F42, K43, F44, Y45, E61, E62, E68, K64, P65, V69, L72, and Y107 is further mutated to

an unnatural amino acid. In some embodiments, the unnatural amino acid comprises N6-azidoethoxy-L-lysine (AzK), N6-propargylethoxy-L-lysine (PraK), BCN-L-lysine, norbornene lysine, TCO-lysine, methyltetrazine lysine, allyloxycarbonyllysine, 2-amino-8-oxononanoic acid, 2-amino-8-oxooctanoic acid, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, 2-amino-8-oxononanoic acid, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphotyrosine, tri-O-acetyl-GlcNAcp-serine, L-phosphoserine, phosphoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, 2-amino-3-(phenylselanyl)propanoic, or selenocysteine. In some embodiments, the IL-2 conjugate has a decreased affinity to IL-2 receptor α (IL-2R α) subunit relative to a wild-type IL-2 polypeptide. In some embodiments, the decreased affinity is about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or greater than 99% decrease in binding affinity to IL-2R α relative to a wild-type IL-2 polypeptide. In some embodiments, the decreased affinity is about 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 500-fold, 1000-fold, or more relative to a wild-type IL-2 polypeptide. In some embodiments, the conjugating moiety impairs or blocks the binding of IL-2 with IL-2R α . In some embodiments, the conjugating moiety comprises a water-soluble polymer. In some embodiments, the additional conjugating moiety comprises a water-soluble polymer. In some embodiments, each of the water-soluble polymers independently comprises polyethylene glycol (PEG), poly(propylene glycol) (PPG), copolymers of ethylene glycol and propylene glycol, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazolines (POZ), poly(N-acryloylmorpholine), or a combination thereof. In some embodiments, each of the water-soluble polymers independently comprises PEG. In some embodiments, the PEG is a linear PEG or a branched PEG. In some embodiments, each of the water-soluble polymers independently comprises a polysaccharide. In some embodiments, the polysaccharide comprises dextran, polysialic acid (PSA), hyaluronic acid (HA), amylose,

heparin, heparan sulfate (HS), dextrin, or hydroxyethyl-starch (HES). In some embodiments, each of the water-soluble polymers independently comprises a glycan. In some embodiments, each of the water-soluble polymers independently comprises polyamine. In some embodiments, the conjugating moiety comprises a protein. In some embodiments, the additional conjugating moiety comprises a protein. In some embodiments, each of the proteins independently comprises an albumin, a transferrin, or a transthyretin. In some embodiments, each of the proteins independently comprises an Fc portion. In some embodiments, each of the proteins independently comprises an Fc portion of IgG. In some embodiments, the conjugating moiety comprises a polypeptide. In some embodiments, the additional conjugating moiety comprises a polypeptide. In some embodiments, each of the polypeptides independently comprises a XTEN peptide, a glycine-rich homoamino acid polymer (HAP), a PAS polypeptide, an elastin-like polypeptide (ELP), a CTP peptide, or a gelatin-like protein (GLK) polymer. In some embodiments, the isolated and purified IL-2 polypeptide is modified by glutamylation. In some embodiments, the conjugating moiety is directly bound to the isolated and purified IL-2 polypeptide. In some embodiments, the conjugating moiety is indirectly bound to the isolated and purified IL-2 polypeptide through a linker. In some embodiments, the linker comprises a homobifunctional linker. In some embodiments, the homobifunctional linker comprises Lomant's reagent dithiobis (succinimidylpropionate) DSP, 3/3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl)suberate (BS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo DST), ethylene glycobis(succinimidylsuccinate) (EGS), disuccinimidyl glutarate (DSG), N,N'-disuccinimidyl carbonate (DSC), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS), dimethyl-3,3'-dithiobispropionimidate (DTBP), 1,4-di-(3'-(2'-pyridyldithio)propionamido)butane (DPDPB), bismaleimidohehexane (BMH), aryl halide-containing compound (DFDNB), such as *e.g.* 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene, 4,4'-difluoro-3,3'-dinitrophenylsulfone (DFDNPS), bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED), formaldehyde, glutaraldehyde, 1,4-butanediol diglycidyl ether, adipic acid dihydrazide, carbohydrazide, o-toluidine, 3,3'-dimethylbenzidine, benzidine, α,α'-p-diaminodiphenyl, diiodo-p-xylene sulfonic acid, N,N'-ethylene-bis(iodoacetamide), or N,N'-hexamethylene-bis(iodoacetamide). In some embodiments, the linker comprises a heterobifunctional linker. In some embodiments, the heterobifunctional linker

comprises N-succinimidyl 3-(2-pyridyldithio)propionate (sPDP), long-chain N-succinimidyl 3-(2-pyridyldithio)propionate (LC-sPDP), water-soluble-long-chain N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-sPDP), succinimidylloxycarbonyl- α -methyl- α -(2-pyridyldithio)toluene (sMPT), sulfosuccinimidyl-6-[α -methyl- α -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-sMPT), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBs), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBs), N-succinimidyl(4-iodoacetyl)aminobenzoate (sIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-sIAB), succinimidyl-4-(p-maleimidophenyl)butyrate (sMPB), sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (sulfo-sMPB), N-(γ -maleimidobutyryloxy)succinimide ester (GMBs), N-(γ -maleimidobutyryloxy) sulfosuccinimide ester (sulfo-GMBs), succinimidyl 6-((iodoacetyl)amino)hexanoate (sIAX), succinimidyl 6-[6-(((iodoacetyl)amino)hexanoyl)amino]hexanoate (sIAXX), succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (sIAC), succinimidyl 6-(((4-iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino) hexanoate (sIACX), p-nitrophenyl iodoacetate (NPiA), carbonyl-reactive and sulfhydryl-reactive cross-linkers such as 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH), 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide-8 (M2C2H), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), N-hydroxysuccinimidyl-4-azidosalicylic acid (NHs-AsA), N-hydroxysulfosuccinimidyl-4-azidosalicylic acid (sulfo-NHs-AsA), sulfosuccinimidyl-(4-azidosalicylamido)hexanoate (sulfo-NHs-LC-AsA), sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (sAsD), N-hydroxysuccinimidyl-4-azidobenzoate (HsAB), N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HsAB), N-succinimidyl-6-(4'-azido-2'-nitrophenyl amino)hexanoate (sANPAH), sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-sANPAH), N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOs), sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate (sAND), N-succinimidyl-4(4-azidophenyl)1,3'-dithiopropionate (sADP), N-sulfosuccinimidyl(4-azidophenyl)-1,3'-dithiopropionate (sulfo-sADP), sulfosuccinimidyl 4-(p-azidophenyl)butyrate (sulfo-sAPB), sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3'-dithiopropionate (sAED), sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate (sulfo-sAMCA), p-nitrophenyl diazopyruvate (pNPDP), p-nitrophenyl-

2-diazo-3,3,3-trifluoropropionate (PNP-DTP), 1-(p-azidosalicylamido)-4-(iodoacetamido)butane (AsIB), N-[4-(p-azidosalicylamido)butyl]-3'-(2'-pyridyldithio) propionamide (APDP), benzophenone-4-iodoacetamide, p-azidobenzoyl hydrazide (ABH), 4-(p-azidosalicylamido)butylamine (AsBA), or p-azidophenyl glyoxal (APG). In some embodiments, the linker comprises a cleavable linker, optionally comprising a dipeptide linker. In some embodiments, the dipeptide linker comprises Val-Cit, Phe-Lys, Val-Ala, or Val-Lys. In some embodiments, the linker comprises a non-cleavable linker. In some embodiments, the linker comprises a maleimide group, optionally comprising maleimidocaproyl (mc), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC), or sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC). In some embodiments, the linker further comprises a spacer. In some embodiments, the spacer comprises p-aminobenzyl alcohol (PAB), p-aminobenzyloxycarbonyl (PABC), a derivative, or an analog thereof. In some embodiments, the conjugating moiety is capable of extending the serum half-life of the IL-2 conjugate. In some embodiments, the additional conjugating moiety is capable of extending the serum half-life of the IL-2 conjugate. In some embodiments, the IL-2 form suitable for use in the invention is a fragment of any of the IL-2 forms described herein. In some embodiments, the IL-2 form suitable for use in the invention is pegylated as disclosed in U.S. Patent Application Publication No. US 2020/0181220 A1 and U.S. Patent Application Publication No. US 2020/0330601 A1. In some embodiments, the IL-2 form suitable for use in the invention is an IL-2 conjugate comprising: an IL-2 polypeptide comprising an N6-azidoethoxy-L-lysine (AzK) covalently attached to a conjugating moiety comprising a polyethylene glycol (PEG), wherein: the IL-2 polypeptide comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO:5; and the AzK substitutes for an amino acid at position K35, F42, F44, K43, E62, P65, R38, T41, E68, Y45, V69, or L72 in reference to the amino acid positions within SEQ ID NO:5. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of one residue relative to SEQ ID NO:5. In some embodiments, the IL-2 form suitable for use in the invention lacks IL-2R alpha chain engagement but retains normal binding to the intermediate affinity IL-2R beta-gamma signaling complex. In some embodiments, the IL-2 form suitable for use in the invention is an IL-2 conjugate comprising: an IL-2 polypeptide comprising an N6-azidoethoxy-L-lysine (AzK) covalently attached to a conjugating moiety comprising a polyethylene glycol (PEG), wherein: the IL-2 polypeptide comprises an amino acid sequence

having at least 90% sequence identity to SEQ ID NO:5; and the AzK substitutes for an amino acid at position K35, F42, F44, K43, E62, P65, R38, T41, E68, Y45, V69, or L72 in reference to the amino acid positions within SEQ ID NO:5. In some embodiments, the IL-2 form suitable for use in the invention is an IL-2 conjugate comprising: an IL-2 polypeptide comprising an N6-azidoethoxy-L-lysine (AzK) covalently attached to a conjugating moiety comprising a polyethylene glycol (PEG), wherein: the IL-2 polypeptide comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:5; and the AzK substitutes for an amino acid at position K35, F42, F44, K43, E62, P65, R38, T41, E68, Y45, V69, or L72 in reference to the amino acid positions within SEQ ID NO:5. In some embodiments, the IL-2 form suitable for use in the invention is an IL-2 conjugate comprising: an IL-2 polypeptide comprising an N6-azidoethoxy-L-lysine (AzK) covalently attached to a conjugating moiety comprising a polyethylene glycol (PEG), wherein: the IL-2 polypeptide comprises an amino acid sequence having at least 98% sequence identity to SEQ ID NO:5; and the AzK substitutes for an amino acid at position K35, F42, F44, K43, E62, P65, R38, T41, E68, Y45, V69, or L72 in reference to the amino acid positions within SEQ ID NO:5.

[0002] In some embodiments, an IL-2 form suitable for use in the invention is nemvaleukin alfa, also known as ALKS-4230 (SEQ ID NO:6), which is available from Alkermes, Inc. Nemvaleukin alfa is also known as human interleukin 2 fragment (1-59), variant (Cys¹²⁵>Ser⁵¹), fused via peptidyl linker (⁶⁰GG⁶¹) to human interleukin 2 fragment (62-132), fused via peptidyl linker (¹³³GSGGGS¹³⁸) to human interleukin 2 receptor α -chain fragment (139-303), produced in Chinese hamster ovary (CHO) cells, glycosylated; human interleukin 2 (IL-2) (75-133)-peptide [Cys¹²⁵(51)>Ser]-mutant (1-59), fused via a G₂ peptide linker (60-61) to human interleukin 2 (IL-2) (4-74)-peptide (62-132) and via a GSG₃S peptide linker (133-138) to human interleukin 2 receptor α -chain (IL2R subunit alpha, IL2R α , IL2RA) (1-165)-peptide (139-303), produced in Chinese hamster ovary (CHO) cells, glycoform alfa. The amino acid sequence of nemvaleukin alfa is given in SEQ ID NO:6. In some embodiments, nemvaleukin alfa exhibits the following post-translational modifications: disulfide bridges at positions: 31-116, 141-285, 184-242, 269-301, 166-197 or 166-199, 168-199 or 168-197 (using the numbering in SEQ ID NO:6), and glycosylation sites at positions: N187, N206, T212 using the numbering in SEQ ID NO:6. The preparation and properties of nemvaleukin alfa, as well as additional alternative forms of IL-2 suitable for use in the invention, is described in U.S. Patent Application Publication No. US

2021/0038684 A1 and U.S. Patent No. 10,183,979, the disclosures of which are incorporated by reference herein. In some embodiments, an IL-2 form suitable for use in the invention is a protein having at least 80%, at least 90%, at least 95%, or at least 90% sequence identity to SEQ ID NO:6. In some embodiments, an IL-2 form suitable for use in the invention has the amino acid sequence given in SEQ ID NO:6 or conservative amino acid substitutions thereof. In some embodiments, an IL-2 form suitable for use in the invention is a fusion protein comprising amino acids 24-452 of SEQ ID NO:7, or variants, fragments, or derivatives thereof. In some embodiments, an IL-2 form suitable for use in the invention is a fusion protein comprising an amino acid sequence having at least 80%, at least 90%, at least 95%, or at least 90% sequence identity to amino acids 24-452 of SEQ ID NO:7, or variants, fragments, or derivatives thereof. Other IL-2 forms suitable for use in the present invention are described in U.S. Patent No. 10,183,979, the disclosures of which are incorporated by reference herein. Optionally, in some embodiments, an IL-2 form suitable for use in the invention is a fusion protein comprising a first fusion partner that is linked to a second fusion partner by a mucin domain polypeptide linker, wherein the first fusion partner is IL-1R α or a protein having at least 98% amino acid sequence identity to IL-1R α and having the receptor antagonist activity of IL-R α , and wherein the second fusion partner comprises all or a portion of an immunoglobulin comprising an Fc region, wherein the mucin domain polypeptide linker comprises SEQ ID NO:8 or an amino acid sequence having at least 90% sequence identity to SEQ ID NO:8 and wherein the half-life of the fusion protein is improved as compared to a fusion of the first fusion partner to the second fusion partner in the absence of the mucin domain polypeptide linker.

TABLE 2. Amino acid sequences of interleukins.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:3 recombinant human IL-2 (rhIL-2)	MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL 60 EELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGSE ETTFMCEYAD ETATIVEFLN 120 RWITFCQSII STLT 134
SEQ ID NO:4 Aldesleukin	PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT KFYMPKKAT ELKHLQCLEE 60 ELKPLEEVLN LAQSKNFHLR PRDLISNIN IVLELKGSET TFMCEYADET ATIVEFLNRW 120 ITFSQSIIST LT 132
SEQ ID NO:5 IL-2 form	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML TTFKFYMPKKA TELKHLQCLE 60 EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120 WITFCQSII S TLT 133
SEQ ID NO:6 Nemvaleukin alfa	SKNFHLRPRD LISNINVIVL ELKGSETTFM CEYADETATI VEFLNRWITF SQSIISTLTG 60 GSSSTKKTQL QLEHLLLDLQ MILNGINNYK NPKLTRMLTF KFYMPKKATE LKHLQCLEEE 120 LKPLEEVLNL AQSGGGSEL CDDDPPEIPH ATKAMAYKE GTMLNCECKR GFRRIKSGSL 180 YMLCTGNSSH SSWDNQCQCT SSATRNNTKQ VTPQPEEQKE RKTTEMQSPM QPVDQASLPG 240 HCREPPWEN EATERIYHFV VGQMVYYQCV QGYRALHRGP AESVCKMTHG KTRWTQPQLI 300 CTG 303
SEQ ID NO:7	MDAMKRGLCC VLLLCGAVFV SARRPSGRKS SKMQAFRIWD VNQKTFYLRN NQLVAGYLQG 60

IL-2 form	PNVNLEEKID VVPIEPHALF LGIHGGKMCL SCVKSGDETR LQLEAVNITD LSENKKQDKR 120 FAFIRSDSGP TTSFESAACP GWFLCTAMEA DQPVSLTNMP DEGVMVTKFY FQDESGSGG 180 ASSESSASSD GPHPVITESR ASSESSASSD GPHPVITESR EPKSSDKTHT CPPCPAPELL 240 GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ 300 YNSTYRVVSV LTVLHQDWLN GKEYCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR 360 EEMTKNQVSL TCLVKGFYPS DIAVEWESNG OPENNYKTP PVLDSDGSFF LYSKLTVDKS 420 RWQQGNVFSC SVMHEALHNNH YTKQSLSLSP GK 452
SEQ ID NO: 8 mucin domain polypeptide	SESSASSDGP HPVITP 16
SEQ ID NO: 9 recombinant human IL-4 (rhIL-4)	MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCAA TVLRQFYSHH 60 EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLTKI 120 MREKYSKCSS 130
SEQ ID NO: 10 recombinant human IL-7 (rhIL-7)	MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA 60 ARKLRQFLKM NSTGDFDLHL LKVGEGTTL LNCTGQVQGR KPAALGAEAP TKSLEENKSL 120 KEQKKLNDLC FLKRLLEIK TCWNKILMGT KEH 153
SEQ ID NO: 11 recombinant human IL-15 (rhIL-15)	MNWVNVISDL KKIEDLIQSM HIDAITYTES DVHPSCVTA MKCFLELQV ISLESGDASI 60 HDTVENLIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS 115
SEQ ID NO: 12 recombinant human IL-21 (rhIL-21)	MQDRHMIRMRLQIDIVDLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG 60 NNERIINVS I KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLQKMIHQ 120 HLSSRTHGSE DS 132

[00281] In some embodiments, an IL-2 form suitable for use in the invention includes a antibody cytokine engrafted protein comprises a heavy chain variable region (V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L, wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain variable region (V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L, wherein the IL-2 molecule is a mutein, and wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the IL-2 regimen comprises administration of an antibody described in U.S. Patent Application Publication No. US 2020/0270334 A1, the disclosures of which are incorporated by reference herein. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain variable region (V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L, wherein the IL-2 molecule is a mutein, wherein the antibody cytokine

engrafted protein preferentially expands T effector cells over regulatory T cells, and wherein the antibody further comprises an IgG class heavy chain and an IgG class light chain selected from the group consisting of: a IgG class light chain comprising SEQ ID NO:39 and a IgG class heavy chain comprising SEQ ID NO:38; a IgG class light chain comprising SEQ ID NO:37 and a IgG class heavy chain comprising SEQ ID NO:29; a IgG class light chain comprising SEQ ID NO:39 and a IgG class heavy chain comprising SEQ ID NO:29; and a IgG class light chain comprising SEQ ID NO:37 and a IgG class heavy chain comprising SEQ ID NO:38.

[00282] In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into HCDR1 of the V_H, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into HCDR2 of the V_H, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into HCDR3 of the V_H, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into LCDR1 of the V_L, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into LCDR2 of the V_L, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into LCDR3 of the V_L, wherein the IL-2 molecule is a mutein.

[00283] The insertion of the IL-2 molecule can be at or near the N-terminal region of the CDR, in the middle region of the CDR or at or near the C-terminal region of the CDR. In some embodiments, the antibody cytokine engrafted protein comprises an IL-2 molecule incorporated into a CDR, wherein the IL2 sequence does not frameshift the CDR sequence. In some embodiments, the antibody cytokine engrafted protein comprises an IL-2 molecule incorporated into a CDR, wherein the IL-2 sequence replaces all or part of a CDR sequence. The replacement by the IL-2 molecule can be the N-terminal region of the CDR, in the middle region of the CDR or at or near the C-terminal region the CDR. A replacement by the IL-2 molecule can be as few as one or two amino acids of a CDR sequence, or the entire CDR sequences.

[00284] In some embodiments, an IL-2 molecule is engrafted directly into a CDR without a peptide linker, with no additional amino acids between the CDR sequence and the IL-2 sequence. In some embodiments, an IL-2 molecule is engrafted indirectly into a CDR with a peptide linker, with one or more additional amino acids between the CDR sequence and the IL-2 sequence.

[00285] In some embodiments, the IL-2 molecule described herein is an IL-2 mutein. In some instances, the IL-2 mutein comprising an R67A substitution. In some embodiments, the IL-2 mutein comprises the amino acid sequence SEQ ID NO:14 or SEQ ID NO:15. In some embodiments, the IL-2 mutein comprises an amino acid sequence in Table 1 in U.S. Patent Application Publication No. US 2020/0270334 A1, the disclosure of which is incorporated by reference herein.

[00286] In some embodiments, the antibody cytokine engrafted protein comprises an HCDR1 selected from the group consisting of SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:22 and SEQ ID NO:25. In some embodiments, the antibody cytokine engrafted protein comprises an HCDR1 selected from the group consisting of SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:13 and SEQ ID NO:16. In some embodiments, the antibody cytokine engrafted protein comprises an HCDR1 selected from the group consisting of HCDR2 selected from the group consisting of SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:23, and SEQ ID NO:26. In some embodiments, the antibody cytokine engrafted protein comprises an HCDR3 selected from the group consisting of SEQ ID NO:18, SEQ ID NO:21, SEQ ID NO:24, and SEQ ID NO:27. In some embodiments, the antibody cytokine engrafted protein comprises a V_H region comprising the amino acid sequence of SEQ ID NO:28. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain comprising the amino acid sequence of SEQ ID NO:29. In some embodiments, the antibody cytokine engrafted protein comprises a V_L region comprising the amino acid sequence of SEQ ID NO:36. In some embodiments, the antibody cytokine engrafted protein comprises a light chain comprising the amino acid sequence of SEQ ID NO:37. In some embodiments, the antibody cytokine engrafted protein comprises a V_H region comprising the amino acid sequence of SEQ ID NO:28 and a V_L region comprising the amino acid sequence of SEQ ID NO:36. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:29 and a light chain region comprising the amino acid sequence of SEQ ID NO:37. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:29 and a light chain region comprising the amino acid sequence of SEQ ID NO:39. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:38 and a light chain region comprising the amino acid sequence of SEQ ID NO:37. In some embodiments, the antibody

cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:38 and a light chain region comprising the amino acid sequence of SEQ ID NO:39. In some embodiments, the antibody cytokine engrafted protein comprises IgG.IL2F71A.H1 or IgG.IL2R67A.H1 of U.S. Patent Application Publication No. 2020/0270334 A1, or variants, derivatives, or fragments thereof, or conservative amino acid substitutions thereof, or proteins with at least 80%, at least 90%, at least 95%, or at least 98% sequence identity thereto. In some embodiments, the antibody components of the antibody cytokine engrafted protein described herein comprise immunoglobulin sequences, framework sequences, or CDR sequences of palivizumab. In some embodiments, the antibody cytokine engrafted protein described herein has a longer serum half-life than a wild-type IL-2 molecule such as, but not limited to, aldesleukin or a comparable molecule. In some embodiments, the antibody cytokine engrafted protein described herein has a sequence as set forth in Table 3.

TABLE 3: Sequences of exemplary palivizumab antibody-IL-2 engrafted proteins

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:13 IL-2	MYRMLQLLSCT ALSALVTNS APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML 60 TFKFYMPKKA TELKHLQCLE EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE 120 TTFMCEYADE TATIVEFLNR WITFCQSIIS TLT 153
SEQ ID NO:14 IL-2 mutein	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTAML TFKFYMPKKA TELKHLQCLE 60 EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120 WITFCQSIIS TLT 133
SEQ ID NO:15 IL-2 mutein	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML TAKFYMPKKA TELKHLQCLE 60 EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120 WITFCQSIIS TLT 133
SEQ ID NO:16 HCDR1_IL-2	GFSLAPTSSS TTKTQLQLEH LLLDLQMILN GINNYKNPKL TAMLTFKFYM PKKATELKHL 60 QCLEEELKPL EEVLNLAQSK NFHLRPRDLI SNINVIVLEL KGSETTFMCE YADETATIVE 120 FLNRWITFCQ SIISTLTSTS GMSVG 145
SEQ ID NO:17 HCDR2	DIWWDDKKDY NPSLKS 16
SEQ ID NO:18 HCDR3	SMITNWFYFDV 10
SEQ ID NO:19 HCDR1_IL-2 kabat	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTAML TFKFYMPKKA TELKHLQCLE 60 EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120 WITFCQSIIS TLTSTSGMSV G 141
SEQ ID NO:20 HCDR2 kabat	DIWWDDKKDY NPSLKS 16
SEQ ID NO:21 HCDR3 kabat	SMITNWFYFDV 10
SEQ ID NO:22 HCDR1_IL-2 clothia	GFSLAPTSSS TTKTQLQLEH LLLDLQMILN GINNYKNPKL TAMLTFKFYM PKKATELKHL 60 QCLEEELKPL EEVLNLAQSK NFHLRPRDLI SNINVIVLEL KGSETTFMCE YADETATIVE 120 FLNRWITFCQ SIISTLTSTS GM 142

SEQ ID NO:23	WWDDK	5	
HCDR2 clothia			
SEQ ID NO:24	SMITNWFYFDV 10		
HCDR3 clothia			
SEQ ID NO:25	GFSLAPTSSS TKKTQLQLEH LLLDLQMILN GINNYKNPKL TAMLTFKFYM PKKATELKHL		
HCDR1_IL-2 IMGT	60		
	QCLEEELKPL EEVLNLAQSK NFHLRPRDLI SNINVIVLEL KGSETTFMCE YADETATIVE		
	120		
	FLNRWITFCQ SIISTLTSTS GMS 143		
SEQ ID NO:26	IWWDDKK	7	
HCDR2 IMGT			
SEQ ID NO:27	ARSMITNWFY DV 12		
HCDR3 IMGT			
SEQ ID NO:28	QVTLRESGPA LVKPTQTL TL TCTFSGFSLA PTSSSTKKTQ LQLEHLLLDL QMILNGINNY		
V _H	60		
	KNPKLTAMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISINIV		
	120		
	IVLELKGSET TFMCEYADET ATIVEFLNRW ITFCQSIIST LTSTSGMSVG WIRQPPGKAL		
	180		
	EWLADIWDD KKDYNPSLKS RL TISKDTSK NQVVLKVTNM DPADTATYYC		
	ARSMITNWFY 240		
	DVWGAGTTVT VSS 253		
SEQ ID NO:29	QMILNGINNY KNPKL TAML T FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR		
Heavy chain	60		
	PRDLISINIV IVLELKGSET TFMCEYADET ATIVEFLNRW ITFCQSIIST LTSTSGMSVG		
	120		
	WIRQPPGKAL EWLADIWDD KKDYNPSLKS RL TISKDTSK NQVVLKVTNM		
	DPADTATYYC 180		
	ARSMITNWFY DVWGAGTTVT VSSASTGPS VFPLAPSSKS TSGGTAALGC LVKDYFPEPV		
	240		
	TVSWNSGALT SGVHTFPAVL QSSGLYSLSS VVTPSSSLG TQTYICNVNH KPSNTKVDKR		
	300		
	VEPKSCDKTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV AVSHEDPEVK		
	360		
	FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS		
	NKALAAPIEK 420		
	TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT		
	480		
	PPVLDSDGSF FLYSKL TVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLG PGK 533		
SEQ ID NO:30	KAQLSVGYMH 10		
LCDR1 kabat			
SEQ ID NO:31	DTSKLAS	7	
LCDR2 kabat			
SEQ ID NO:32	FQSGGYPT 9		
LCDR3 kabat			
SEQ ID NO:33	QLSVGY	6	
LCDR1 chothia			
SEQ ID NO:34	DTS	3	
LCDR2 chothia			
SEQ ID NO:35	GSGYPF	6	
LCDR3 chothia			
SEQ ID NO:36	DIQMTQSPST LSASVGDRVT ITCAQLSVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR 60		
V _L	FSGSGSGTEF TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKLEIK 106		
SEQ ID NO:37	DIQMTQSPST LSASVGDRVT ITCAQLSVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR 60		
Light chain	FSGSGSGTEF TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKLEIKRTVA APSVFIFPPS 120		
	DEQLKSGTAS VVCLNNFYF REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSTLT 180		
	SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC 213		
SEQ ID NO:38	QVTLRESGPA LVKPTQTL TL TCTFSGFSLA PTSSSTKKTQ LQLEHLLLDL QMILNGINNY 60		
Light chain	KNPKLTRMLT AKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISINIV 120		
	IVLELKGSET TFMCEYADET ATIVEFLNRW ITFCQSIIST LTSTSGMSVG WIRQPPGKAL 180		
	EWLADIWDD KKDYNPSLKS RL TISKDTSK NQVVLKVTNM DPADTATYYC ARSMITNWFY 240		
	DVWGAGTTVT VSSASTGPS VFPLAPSSKS TSGGTAALGC LVKDYFPEPV TVSWNSGALT 300		
	SGVHTFPAVL QSSGLYSLSS VVTPSSSLG TQTYICNVNH KPSNTKVDKR VEPKSCDKTH 360		
	TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV AVSHEDPEVK FNWYVDGVEV 420		
	HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALAAPIEK TISKAKGQPR 480		
	EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF 540		
	FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLG PGK 583		
SEQ ID NO:39	DIQMTQSPST LSASVGDRVT ITCAQLSVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR 60		
Light chain	FSGSGSGTEF TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKLEIKRTVA APSVFIFPPS 120		
	DEQLKSGTAS VVCLNNFYF REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSTLT 180		

SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC

213

[00287] 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:9).

[00288] 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:10).

[00289] 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:11).

[00290] The term “IL-21” (also referred to herein as “IL21”) refers to the pleiotropic cytokine protein known as interleukin-21, and includes all forms of IL-21 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-21 is described, *e.g.*, in Spolski and Leonard, *Nat. Rev. Drug. Disc.* **2014**, *13*, 379-95, the disclosure of which is incorporated by reference herein. IL-21 is primarily produced by natural killer T cells and activated human CD4⁺ T cells. Recombinant human IL-21 is a single, non-glycosylated polypeptide chain containing 132 amino acids with a molecular mass of 15.4 kDa. Recombinant human IL-21 is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-408-b) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-21 recombinant protein, Cat. No. 14-8219-80). The amino acid sequence of recombinant human IL-21 suitable for use in the invention is given in Table 2 (SEQ ID NO:21).

[00291] The term “IL-15R agonist” (also referred to herein as “IL-15 agonist”) refers to a molecule that activates the IL-15 signalling pathway through binding to the IL-15 receptor (IL-15R) β and common γ (γ C) subunits. IL-15 functions through a trans-presentation mechanism in which IL-15 is presented in a complex with a membrane-bound α -subunit of IL-15 receptor (IL-15R α) on the surface of a dendritic or other cell, which complex interacts with the IL-15R β and γ C subunits expressed on NK, NKT or T cells. *See* Stonier and Schluns, *Immunol Lett.* **2010**, *127*, 85–92, the disclosure of which is incorporated by reference herein. IL-15 agonists are described, *e.g.*, in Wu, *J Mol Genet Med.* **2013**, *7*, 85, the disclosure of which is incorporated by reference herein. In some embodiments, an IL-15R agonist may be a recombinant IL-15 molecule. In some embodiments, an IL-15R agonist may be a mimetic of the IL-15/IL-15R α complex presented on a cell surface, for example, a heterodimeric complex or a fusion protein that comprises an IL-15 wildtype or mutant (*e.g.*, N72D, D30N, E64Q, N65D) molecule and partial or whole extracellular domain of IL-15R α , *e.g.*, a soluble IL-15R α , the sushi domain of IL-15R α , etc., optionally linked to one or more Fc domains. In some embodiments, an IL-15R agonist may be a modified IL-15 molecule, *e.g.*, an IL-15 mutant molecule (*e.g.*, N72D, D30N,

E64Q, N65D), an IL-15 with site-specific glycosylation(s), etc., with improved characteristics, e.g., prolonged half-life, increased affinity to IL-15R, etc.

[00292] When “an anti-tumor effective amount”, “a tumor-inhibiting effective amount”, or “therapeutic amount” is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the tumor infiltrating lymphocytes (e.g. secondary TILs or genetically modified cytotoxic lymphocytes) described herein may be administered at a dosage of 10^4 to 10^{11} cells/kg body weight (e.g., 10^5 to 10^6 , 10^5 to 10^{10} , 10^5 to 10^{11} , 10^6 to 10^{10} , 10^6 to 10^{11} , 10^7 to 10^{11} , 10^7 to 10^{10} , 10^8 to 10^{11} , 10^8 to 10^{10} , 10^9 to 10^{11} , or 10^9 to 10^{10} cells/kg body weight), including all integer values within those ranges. TILs (including in some cases, genetically modified cytotoxic lymphocytes) compositions may also be administered multiple times at these dosages. The TILs (including, in some cases, genetically engineered TILs) can be administered by using infusion techniques that are commonly known in immunotherapy (see, e.g., Rosenberg, *et al.*, *New Eng. J. of Med.* **1988**, 319, 1676). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

[00293] The term “hematological malignancy”, “hematologic malignancy” or terms of correlative meaning refer to mammalian cancers and tumors of the hematopoietic and lymphoid tissues, including but not limited to tissues of the blood, bone marrow, lymph nodes, and lymphatic system. Hematological malignancies are also referred to as “liquid tumors.” Hematological malignancies include, but are not limited to, acute lymphoblastic leukemia (ALL), chronic lymphocytic lymphoma (CLL), small lymphocytic lymphoma (SLL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), multiple myeloma, 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.

[00294] The term “liquid tumor” refers to an abnormal mass of cells that is fluid in nature. Liquid tumor cancers include, but are not limited to, leukemias, myelomas, and lymphomas, as well as other hematological malignancies. TILs obtained from liquid tumors may also be referred

to herein as marrow infiltrating lymphocytes (MILs). TILs obtained from liquid tumors, including liquid tumors circulating in peripheral blood, may also be referred to herein as PBLs. The terms MIL, TIL, and PBL are used interchangeably herein and differ only based on the tissue type from which the cells are derived.

[00295] 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.

[00296] In some embodiments, 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 some embodiments, 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 some embodiments, 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.

[00297] 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.

[00298] The term “effective amount” or “therapeutically effective amount” refers to that amount of a compound or combination of compounds as described herein that is sufficient to effect the intended application including, but not limited to, disease treatment. A therapeutically effective amount may vary depending upon the intended application (in vitro or in vivo), or the subject and disease condition being treated (*e.g.*, the weight, age and gender of the subject), the severity of the disease condition, or the manner of administration. The term also applies to a dose that will induce a particular response in target cells (*e.g.*, the reduction of platelet adhesion and/or cell migration). The specific dose will vary depending on the particular compounds chosen, the dosing regimen to be followed, whether the compound is administered in combination with other compounds, timing of administration, the tissue to which it is administered, and the physical delivery system in which the compound is carried.

[00299] The terms “treatment”, “treating”, “treat”, and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease.

“Treatment”, as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, *i.e.*, arresting its development or progression; and (c) relieving the disease, *i.e.*, causing regression of the disease and/or relieving one or more disease symptoms. “Treatment” is also meant to encompass delivery of an agent in order to provide for a pharmacologic effect, even in the absence of a disease or condition. For example, “treatment” encompasses delivery of a composition that can elicit an immune response or confer immunity in the absence of a disease condition, *e.g.*, in the case of a vaccine.

[00300] As used herein, the term “immune checkpoint inhibitor (ICI)” has its general meaning in the art and refers to any compound inhibiting the function of an immune inhibitory checkpoint protein. As used herein the term “immune checkpoint protein” has its general meaning in the art and refers to a molecule that is expressed by T cells and that either turns up a signal (stimulatory checkpoint molecules) or turns down a signal (inhibitory checkpoint molecules). Immune checkpoint molecules are recognized in the art to constitute elements of immune checkpoint pathways similar to the CTLA-4 and PD-1 dependent pathways (see *e.g.*, Pardoll, 2012. Nature

Rev Cancer 12:252-264; Mellman et al., 2011. Nature 480:480-489). Examples of inhibitory checkpoint molecules include A2AR, B7-H3, B7-H4, CD277, IDO, KIR, VISTA, PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF β , PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, TET2, BAFF (BR3), CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, TOX, SOCS1, ANKRD11, and BCOR. For example, immune checkpoint genes that may be silenced or inhibited in TILs of the present invention may be selected from the group comprising PD-1, CTLA-4, LAG-3, TIM-3, CISH, CBL-B, TIGIT, TET2, TGF β , and PKA. BAFF (BR3) is described in Bloom, *et al.*, *J. Immunother.*, **2018**, in press. According to another example, immune checkpoint genes that may be silenced or inhibited in TILs of the present invention may be selected from the group comprising PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, TET2, CISH, TGF β 2, PRA, CBLB, BAFF (BR3), and combinations thereof.

[00301] Inhibition includes reduction of function and full blockade. Preferred immune checkpoint inhibitors are antibodies that specifically recognize immune checkpoint proteins. A number of immune checkpoint inhibitors are known and analogous to these known immune checkpoint protein inhibitors, alternative immune checkpoint inhibitors may be developed in the (near) future. The immune checkpoint inhibitors include peptides, antibodies, nucleic acid molecules and small molecules.

[00302] The terms “non-myeloablative chemotherapy,” “non-myeloablative lymphodepletion,” “NMALD,” “NMA LD,” “NMA-LD,” and any variants of the foregoing, are used interchangeably to indicate a chemotherapeutic regimen designed to deplete the patient’s lymphoid immune cells while avoiding depletion of the patient’s myeloid immune cells. Typically, the patient receives a course of non-myeloablative chemotherapy prior to the administration of tumor infiltrating lymphocytes to the patient as described herein.

[00303] The term “heterologous” when used with reference to portions of a nucleic acid or protein indicates that the nucleic acid or protein comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically

recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, *e.g.*, a promoter from one source and a coding region from another source, or coding regions from different sources. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (*e.g.*, a fusion protein).

[00304] The terms “sequence identity,” “percent identity,” and “sequence percent identity” (or synonyms thereof, *e.g.*, “99% identical”) in the context of two or more nucleic acids or polypeptides, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned (introducing gaps, if necessary) for maximum correspondence, not considering any conservative amino acid substitutions as part of the sequence identity. The percent identity can be measured using sequence comparison software or algorithms or by visual inspection. Various algorithms and software are known in the art that can be used to obtain alignments of amino acid or nucleotide sequences. Suitable programs to determine percent sequence identity include for example the BLAST suite of programs available from the U.S. Government’s National Center for Biotechnology Information BLAST web site. Comparisons between two sequences can be carried using either the BLASTN or BLASTP algorithm. BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. ALIGN, ALIGN-2 (Genentech, South San Francisco, California) or MegAlign, available from DNASTAR, are additional publicly available software programs that can be used to align sequences. One skilled in the art can determine appropriate parameters for maximal alignment by particular alignment software. In certain embodiments, the default parameters of the alignment software are used.

[00305] As used herein, the term “variant” encompasses but is not limited to antibodies or fusion proteins which comprise an amino acid sequence which differs from the amino acid sequence of a reference antibody by way of one or more substitutions, deletions and/or additions at certain positions within or adjacent to the amino acid sequence of the reference antibody. The variant may comprise one or more conservative substitutions in its amino acid sequence as compared to the amino acid sequence of a reference antibody. Conservative substitutions may involve, *e.g.*, the substitution of similarly charged or uncharged amino acids. The variant retains the ability to specifically bind to the antigen of the reference antibody. The term variant also includes pegylated antibodies or proteins.

[00306] By “tumor infiltrating lymphocytes” or “TILs” herein is meant a population of cells originally obtained as white blood cells that have left the bloodstream of a subject and migrated into a tumor. TILs include, but are not limited to, CD8⁺ cytotoxic T cells (lymphocytes), Th1 and Th17 CD4⁺ T cells, natural killer cells, dendritic cells and M1 macrophages. TILs include both primary and secondary TILs. “Primary TILs” are those that are obtained from patient tissue samples as outlined herein (sometimes referred to as “freshly harvested”), and “secondary TILs” are any TIL cell populations that have been expanded or proliferated as discussed herein, including, but not limited to bulk TILs, expanded TILs (“REP TILs”) as well as “reREP TILs” as discussed herein. reREP TILs can include for example second expansion TILs or second additional expansion TILs (such as, for example, those described in Step G of Figure 5A and Figure 5C, Step I of Figure 5B, and/or Step H of Figure 5D), , including TILs referred to as reREP TILs).

[00307] 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. TILs may further be characterized by potency – for example, TILs may be considered potent if, for example, interferon (IFN) release is greater than about 50 pg/mL, greater than about 100 pg/mL, greater than about 150 pg/mL, or greater than about 200 pg/mL. TILs may be considered potent if, for example, interferon (IFN γ) release is greater than about 50 pg/mL, greater than about 100 pg/mL, greater than about 150 pg/mL, or greater than about 200 pg/mL, greater than about 300 pg/mL, greater than about 400 pg/mL, greater than about 500 pg/mL, greater than about 600 pg/mL, greater than about 700 pg/mL, greater than about 800 pg/mL, greater than about 900 pg/mL, greater than about 1000 pg/mL.

[00308] The term “deoxyribonucleotide” encompasses natural and synthetic, unmodified and modified deoxyribonucleotides. Modifications include changes to the sugar moiety, to the base moiety and/or to the linkages between deoxyribonucleotide in the oligonucleotide.

[00309] The term “RNA” defines a molecule comprising at least one ribonucleotide residue. The term “ribonucleotide” defines a nucleotide with a hydroxyl group at the 2' position of a b-D-

ribofuranose moiety. The term RNA includes double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Nucleotides of the RNA molecules described herein may also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

[00310] The terms “pharmaceutically acceptable carrier” or “pharmaceutically acceptable excipient” are intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and inert ingredients. The use of such pharmaceutically acceptable carriers or pharmaceutically acceptable excipients for active pharmaceutical ingredients is well known in the art. Except insofar as any conventional pharmaceutically acceptable carrier or pharmaceutically acceptable excipient is incompatible with the active pharmaceutical ingredient, its use in therapeutic compositions of the invention is contemplated. Additional active pharmaceutical ingredients, such as other drugs, can also be incorporated into the described compositions and methods.

[00311] The terms “about” and “approximately” mean within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, more preferably still within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the terms “about” or “approximately” depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art. Moreover, as used herein, the terms “about” and “approximately” mean that dimensions, sizes, formulations, parameters, shapes and other quantities and characteristics are not and need not be exact, but may be approximate and/or larger or smaller, as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art. In general, a dimension, size, formulation, parameter, shape or other quantity or characteristic is “about” or “approximate” whether or not expressly stated to be such. It is noted that embodiments of very different sizes, shapes and dimensions may employ the described arrangements.

[00312] The transitional terms “comprising,” “consisting essentially of,” and “consisting of,” when used in the appended claims, in original and amended form, define the claim scope with respect to what unrecited additional claim elements or steps, if any, are excluded from the scope of the claim(s). The term “comprising” is intended to be inclusive or open-ended and does not exclude any additional, unrecited element, method, step or material. The term “consisting of” excludes any element, step or material other than those specified in the claim and, in the latter instance, impurities ordinary associated with the specified material(s). The term “consisting essentially of” limits the scope of a claim to the specified elements, steps or material(s) and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. All compositions, methods, and kits described herein that embody the present invention can, in alternate embodiments, be more specifically defined by any of the transitional terms “comprising,” “consisting essentially of,” and “consisting of.”

[00313] 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, CL. 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.

[00314] The term “antigen” refers to a substance that induces an immune response. In some embodiments, an antigen is a molecule capable of being bound by an antibody or a TCR if presented by major histocompatibility complex (MHC) molecules. The term “antigen”, as used herein, also encompasses T cell epitopes. An antigen is additionally capable of being recognized by the immune system. In some embodiments, an antigen is capable of inducing a humoral immune response or a cellular immune response leading to the activation of B lymphocytes and/or T lymphocytes. In some cases, this may require that the antigen contains or is linked to a Th cell epitope. An antigen can also have one or more epitopes (*e.g.*, B- and T-epitopes). In some embodiments, an antigen will preferably react, typically in a highly specific and selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be induced by other antigens.

[00315] The terms “monoclonal antibody,” “mAb,” “monoclonal antibody composition,” or their plural forms refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Monoclonal antibodies specific to certain receptors can be made using knowledge and skill in the art of injecting test subjects with suitable antigen and then isolating hybridomas expressing antibodies having the desired sequence or functional characteristics. DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

[00316] The terms “antigen-binding portion” or “antigen-binding fragment” of an antibody (or simply “antibody portion” or “fragment”), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term “antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and

CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment (Ward, *et al.*, *Nature*, **1989**, 341, 544-546), which may consist of a V_H or a V_L domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules known as single chain Fv (scFv); see, *e.g.*, Bird, *et al.*, *Science* **1988**, 242, 423-426; and Huston, *et al.*, *Proc. Natl. Acad. Sci. USA* **1988**, 85, 5879-5883). Such scFv antibodies are also intended to be encompassed within the terms “antigen-binding portion” or “antigen-binding fragment” of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. In some embodiments, a scFv protein domain comprises a V_H portion and a V_L portion. A scFv molecule is denoted as either V_L-L-V_H if the V_L domain is the N-terminal part of the scFv molecule, or as V_H-L-V_L if the V_H domain is the N-terminal part of the scFv molecule. Methods for making scFv molecules and designing suitable peptide linkers are described in U.S. Pat. No. 4,704,692, U.S. Pat. No. 4,946,778, R. Raag and M. Whitlow, “Single Chain Fvs.” FASEB Vol 9:73-80 (1995) and R. E. Bird and B. W. Walker, Single Chain Antibody Variable Regions, TIBTECH, Vol 9: 132-137 (1991), the disclosures of which are incorporated by reference herein.

[00317] 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.

[00318] 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 some embodiments, 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.

[00319] The term “recombinant human antibody”, as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (such as a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[00320] As used herein, “isotype” refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by the heavy chain constant region genes.

[00321] 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.”

[00322] The term “human antibody derivatives” refers to any modified form of the human antibody, including a conjugate of the antibody and another active pharmaceutical ingredient or antibody. The terms “conjugate,” “antibody-drug conjugate,” “ADC,” or “immunoconjugate”

refers to an antibody, or a fragment thereof, conjugated to another therapeutic moiety, which can be conjugated to antibodies described herein using methods available in the art.

[00323] The terms “humanized antibody,” “humanized antibodies,” and “humanized” are intended to refer to antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences. Additional framework region modifications may be made within the human framework sequences. Humanized forms of non-human (for example, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a 15 hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones, *et al.*, *Nature* **1986**, 321, 522-525; Riechmann, *et al.*, *Nature* **1988**, 332, 323-329; and Presta, *Curr. Op. Struct. Biol.* **1992**, 2, 593-596. The antibodies described herein may also be modified to employ any Fc variant which is known to impart an improvement (*e.g.*, reduction) in effector function and/or FcR binding. The Fc variants may include, for example, any one of the amino acid substitutions disclosed in International Patent Application Publication Nos. WO 1988/07089 A1, WO 1996/14339 A1, WO 1998/05787 A1, WO 1998/23289 A1, WO 1999/51642 A1, WO 99/58572 A1, WO 2000/09560 A2, WO 2000/32767 A1, WO 2000/42072 A2, WO 2002/44215 A2, WO 2002/060919 A2, WO 2003/074569 A2, WO 2004/016750 A2, WO 2004/029207 A2, WO 2004/035752 A2, WO 2004/063351 A2, WO 2004/074455 A2, WO 2004/099249 A2, WO 2005/040217 A2, WO 2005/070963 A1, WO 2005/077981 A2, WO 2005/092925 A2, WO

2005/123780 A2, WO 2006/019447 A1, WO 2006/047350 A2, and WO 2006/085967 A2; and U.S. Patent Nos. 5,648,260; 5,739,277; 5,834,250; 5,869,046; 6,096,871; 6,121,022; 6,194,551; 6,242,195; 6,277,375; 6,528,624; 6,538,124; 6,737,056; 6,821,505; 6,998,253; and 7,083,784; the disclosures of which are incorporated by reference herein.

[00324] 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.

[00325] 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.

[00326] 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.

[00327] “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 and U.S. Patent No. 5,824,778, the disclosures of each of which are incorporated by reference herein.

[00328] The term “biosimilar” means a biological product, including a monoclonal antibody or protein, that is highly similar to a U.S. licensed reference biological product notwithstanding minor differences in clinically inactive components, and for which there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product. Furthermore, a similar biological or “biosimilar” medicine is a biological medicine that is similar to another biological medicine that has already been authorized for use by the European Medicines Agency. The term “biosimilar” is also used synonymously by other national and regional regulatory agencies. Biological products or biological medicines are medicines that are made by or derived from a biological source, such as a bacterium or yeast. They can consist of relatively small molecules such as human insulin or erythropoietin, or complex molecules such as monoclonal antibodies. For example, if the reference IL-2 protein is aldesleukin (PROLEUKIN), a protein approved by drug regulatory authorities with reference to aldesleukin is a “biosimilar to” aldesleukin or is a “biosimilar thereof” of aldesleukin. In Europe, a similar biological or “biosimilar” medicine is a biological medicine that is similar to another biological medicine that has already been authorized for use by the European Medicines Agency (EMA). The relevant legal basis for similar biological applications in Europe is Article 6 of Regulation (EC) No 726/2004 and Article 10(4) of Directive 2001/83/EC, as amended and therefore in Europe, the biosimilar may be authorized, approved for authorization or subject of an application for authorization under Article 6 of Regulation (EC) No 726/2004 and Article 10(4) of Directive 2001/83/EC. The already authorized original biological medicinal product may be referred to as a “reference medicinal product” in Europe. Some of the requirements for a product to be considered a biosimilar are outlined in the CHMP Guideline on Similar Biological Medicinal Products. In addition, product specific guidelines, including guidelines relating to monoclonal antibody biosimilars, are provided on a product-by-product basis by the EMA and published on its website. A biosimilar as described herein may be similar to the reference medicinal product by way of quality characteristics, biological activity, mechanism of action, safety profiles and/or efficacy. In

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

and/or pharmacodynamic (PD) profiles as compared to the reference medicinal product but is still deemed sufficiently similar to the reference medicinal product as to be authorized or considered suitable for authorization. In certain circumstances, the biosimilar exhibits different binding characteristics as compared to the reference medicinal product, wherein the different binding characteristics are considered by a Regulatory Authority such as the EMA not to be a barrier for authorization as a similar biological product. The term “biosimilar” is also used synonymously by other national and regional regulatory agencies.

[00329] The terms “organoid” and “tumoroid,” as used herein are interchangeable and refer to patient-derived microspheres comprising dissociated primary tissues and cells (either normal/healthy or abnormal/diseased/cancerous) and, optionally, a liquid matrix material, wherein the tissue and matrix material form an unpolymerized tissue that is later polymerized to form microspheres that are typically less than about 1000 μm in diameter. In some embodiments, the organoids are less than 900 μm , less than 800 μm , less than 700 μm , less than 600 μm , less than 500 μm in diameter.

[00330] The dissociated primary tissue and/or cells may be freshly biopsied and obtained in any appropriate manner, including mechanical or chemical dissociation (*e.g.*, enzymatic disaggregation) by using one or more enzymes, such as collagenase, trypsin, etc.). The dissociated tissues and/or cells may optionally be treated, selected and / or modified. For example, the cells may be sorted or selected to identify and / or isolate cells having one or more characteristics (*e.g.*, size, morphology, etc.). The cells may be marked (*e.g.*, with one or more markers) that may be used to aid in selection. In some embodiments, the cells may be sorted by well-characterized cell sorting technology, including but not limited to microfluidic cell sorting, fluorescent activated cell sorting, magnetic activated cell sorting, etc.

[00331] The number of dissociated cells may be within a predetermined range, as mentioned above (*e.g.*, between about 1,000 and about 10,000 cells, between about 10,000 and about 100,000 cells, between about 100,000 and about 500,000 cells, between about 500,000 cells and about 1,000,000 cells, between about 1,000,000 cells and about 2,000,000 cells, or between about 2,000,000 cells and about 3,000,000 cells). In some embodiments, one or more organoids or tumoroids may contain about 3,000,000 tumor-derived cells. Any of these methods may be configured as described herein to produce organoids or tumoroids of repeatable size.

[00332] Processes for making organoids and tumoroids are well-characterized and are disclosed in detail in at least the following patent applications, which are hereby incorporated by reference in their entireties: WO 2019/067795 and US 2021/0285054.

II. Methods For Enriching Tumor-Reactive TILs

[00333] Without being limited to any particular theory, it is believed that all expandable TIL subpopulations do not possess equivalent levels of tumor reactivity and that said tumor reactive TIL subpopulations can be distinguished from these “bystander” TIL subpopulations through active selection based upon phenotypic distinctions, such as IFN γ release or protein expression profile of activation/exhaustion markers. It is also believed that such tumor-reactive TIL subpopulations can be enriched in comparison to the “bystander” TIL subpopulations by contacting with an autologous tumor digest or tumor lysate, by contacting with mature dendritic cells that have been previously cultured with tumor antigens – in the form of a tumor digest/tumor lysate or isolated peptides, or by contacting with autologous tumoroids or organoids.

[00334] Therefore, the present disclosure provides a method for enriching a plurality of tumor-reactive TILs. In some embodiments, the method comprises enriching the tumor reactive TILs before the identification of the plurality of tumor reactive TILs. In some embodiments, the enriching step takes place after a first expansion step of the TILs. In some embodiments, the enriching step comprises: (a) co-culture of TILs from the first expansion with autologous tumor digest or tumor lysate; (b) co-culture of TILs from the first expansion with mature dendritic cells (that previously were cultured with autologous tumor antigens—either in the form of a tumor digest/tumor lysate or isolated peptides); or (c) co-culture of the TILs from the first expansion with autologous tumoroids or organoids, such that the tumor reactive TIL population becomes enriched. In some embodiments, the plurality of tumor-reactive TILs is then phenotypically profiled and/or identified. In some embodiments, the enriched plurality of tumor reactive TILs is further expanded by a second expansion step. In some embodiments, the identified plurality of tumor reactive TILs is further expanded by a second expansion step.

[00335] In some embodiments, the steps of profiling TILs to identify tumor-reactive subpopulations and isolation of tumor-reactive populations occur simultaneously, optionally using flow cytometry or other cell separation processes known to one of skill in the art. These

processes include imaging-based methods to separate cell populations based upon cellular morphology. *See generally*, Lin, W., *et al.* (2015). *BMC Immunology*, 16(1), 1-15. Examples of such imaging-based cell separation methods are disclosed in at least the following patent applications, which are herein incorporated by reference in their entireties: WO 2020/037070, US2021/0405022, US 2021/0190669, and US 2020/0150022.

A. Obtaining Patient Tumor Sample

[00336] In general, TILs are initially obtained from a patient tumor sample (“primary TILs”) or from circulating lymphocytes, such as peripheral blood lymphocytes, including peripheral blood lymphocytes having TIL-like characteristics, and are then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, and optionally evaluated for phenotype and metabolic parameters as an indication of TIL health.

[00337] A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of any cancer type, including, but not limited to, breast, pancreatic, prostate, colorectal, lung, brain, renal, stomach, and skin (including but not limited to squamous cell carcinoma, basal cell carcinoma, and melanoma). In some embodiments, the cancer is selected from cervical cancer, head and neck cancer (including, for example, head and neck squamous cell carcinoma (HNSCC)), glioblastoma (GBM), gastrointestinal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, and non-small cell lung carcinoma. In some embodiments, the cancer is melanoma. In some embodiments, useful TILs are obtained from malignant melanoma tumors, as these have been reported to have particularly high levels of TILs.

[00338] 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.

1. Core/Small Biopsy Derived TILs

[00339] In some embodiments, TILs are initially obtained from a patient tumor sample (“primary TILs”) obtained by a core biopsy or similar procedure and then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, and optionally evaluated for phenotype and metabolic parameters.

[00340] In some embodiments, a patient tumor sample may be obtained using methods known in the art, generally via small biopsy, core biopsy, needle biopsy or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. In some embodiments, the sample can be from multiple small tumor samples or biopsies. In some embodiments, the sample can comprise multiple tumor samples from a single tumor from the same patient. In some embodiments, the sample can comprise multiple tumor samples from one, two, three, or four tumors from the same patient. In some embodiments, the sample can comprise multiple tumor samples from multiple tumors from the same patient. The solid tumor may be a lung and/or non-small cell lung carcinoma (NSCLC).

[00341] In general, the cell suspension obtained from the tumor core or fragment is called a “primary cell population” or a “freshly obtained” or a “freshly isolated” cell population. In

certain embodiments, the freshly obtained cell population of TILs is exposed to a cell culture medium comprising antigen presenting cells, IL-2 and OKT-3.

[00342] In some embodiments, if the tumor is metastatic and the primary lesion has been efficiently treated/removed in the past, removal of one of the metastatic lesions may be needed. In some embodiments, the least invasive approach is to remove a skin lesion, or a lymph node on the neck or axillary area when available. In some embodiments, a skin lesion is removed or small biopsy thereof is removed. In some embodiments, a lymph node or small biopsy thereof is removed. In some embodiments, the tumor is a melanoma. In some embodiments, the small biopsy for a melanoma comprises a mole or portion thereof.

[00343] In some embodiments, the small biopsy is a punch biopsy. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin. around a suspicious mole. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin, and a round piece of skin is removed. In some embodiments, the small biopsy is a punch biopsy and round portion of the tumor is removed.

[00344] In some embodiments, the small biopsy is an excisional biopsy. In some embodiments, the small biopsy is an excisional biopsy and the entire mole or growth is removed. In some embodiments, the small biopsy is an excisional biopsy and the entire mole or growth is removed along with a small border of normal-appearing skin.

[00345] In some embodiments, the small biopsy is an incisional biopsy. In some embodiments, the small biopsy is an incisional biopsy and only the most irregular part of a mole or growth is taken. In some embodiments, the small biopsy is an incisional biopsy and the incisional biopsy is used when other techniques can't be completed, such as if a suspicious mole is very large.

[00346] In some embodiments, the small biopsy is a lung biopsy. In some embodiments, the small biopsy is obtained by bronchoscopy. Generally, bronchoscopy, the patient is put under anesthesia, and a small tool goes through the nose or mouth, down the throat, and into the bronchial passages, where small tools are used to remove some tissue. In some embodiments, where the tumor or growth cannot be reached via bronchoscopy, a transthoracic needle biopsy can be employed. Generally, for a transthoracic needle biopsy, the patient is also under anesthesia and a needle is inserted through the skin directly into the suspicious spot to remove a

small sample of tissue. In some embodiments, a transthoracic needle biopsy may require interventional radiology (for example, the use of x-rays or CT scan to guide the needle). In some embodiments, the small biopsy is obtained by needle biopsy. In some embodiments, the small biopsy is obtained endoscopic ultrasound (for example, an endoscope with a light and is placed through the mouth into the esophagus). In some embodiments, the small biopsy is obtained surgically.

[00347] In some embodiments, the small biopsy is a head and neck biopsy. In some embodiments, the small biopsy is an incisional biopsy. In some embodiments, the small biopsy is an incisional biopsy, wherein a small piece of tissue is cut from an abnormal-looking area. In some embodiments, if the abnormal region is easily accessed, the sample may be taken without hospitalization. In some embodiments, if the tumor is deeper inside the mouth or throat, the biopsy may need to be done in an operating room, with general anesthesia. In some embodiments, the small biopsy is an excisional biopsy. In some embodiments, the small biopsy is an excisional biopsy, wherein the whole area is removed. In some embodiments, the small biopsy is a fine needle aspiration (FNA). In some embodiments, the small biopsy is a fine needle aspiration (FNA), wherein a very thin needle attached to a syringe is used to extract (aspirate) cells from a tumor or lump. In some embodiments, the small biopsy is a punch biopsy. In some embodiments, the small biopsy is a punch biopsy, wherein punch forceps are used to remove a piece of the suspicious area.

[00348] In some embodiments, the small biopsy is a cervical biopsy. In some embodiments, the small biopsy is obtained via colposcopy. Generally, colposcopy methods employ the use of a lighted magnifying instrument attached to magnifying binoculars (a colposcope) which is then used to biopsy a small section of the surface of the cervix. In some embodiments, the small biopsy is a conization/cone biopsy. In some embodiments, the small biopsy is a conization/cone biopsy, wherein an outpatient surgery may be needed to remove a larger piece of tissue from the cervix. In some embodiments, the cone biopsy, in addition to helping to confirm a diagnosis, a cone biopsy can serve as an initial treatment.

[00349] The term “solid tumor” refers to an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors may be benign or malignant. The term “solid tumor cancer” refers to malignant, neoplastic, or cancerous solid tumors. Solid tumor cancers include

cancers of the lung. In some embodiments, the cancer is melanoma. In some embodiments, the cancer is non-small cell lung carcinoma (NSCLC). The tissue structure of solid tumors includes interdependent tissue compartments including the parenchyma (cancer cells) and the supporting stromal cells in which the cancer cells are dispersed and which may provide a supporting microenvironment.

[00350] In some embodiments, the sample from the tumor is obtained as a fine needle aspirate (FNA), a core biopsy, a small biopsy (including, for example, a punch biopsy). In some embodiments, sample is placed first into a G-REX-10. In some embodiments, sample is placed first into a G-REX-10 when there are 1 or 2 core biopsy and/or small biopsy samples. In some embodiments, sample is placed first into a G-REX-100 when there are 3, 4, 5, 6, 8, 9, or 10 or more core biopsy and/or small biopsy samples. In some embodiments, sample is placed first into a G-REX-500 when there are 3, 4, 5, 6, 8, 9, or 10 or more core biopsy and/or small biopsy samples.

[00351] The FNA can be obtained from a skin tumor, including, for example, a melanoma. In some embodiments, the FNA is obtained from a skin tumor, such as a skin tumor from a patient with metastatic melanoma. In some cases, the patient with melanoma has previously undergone a surgical treatment.

[00352] The FNA can be obtained from a lung tumor, including, for example, an NSCLC. In some embodiments, the FNA is obtained from a lung tumor, such as a lung tumor from a patient with non-small cell lung cancer (NSCLC). In some cases, the patient with NSCLC has previously undergone a surgical treatment.

[00353] TILs described herein can be obtained from an FNA sample. In some cases, the FNA sample is obtained or isolated from the patient using a fine gauge needle ranging from an 18 gauge needle to a 25 gauge needle. The fine gauge needle can be 18 gauge, 19 gauge, 20 gauge, 21 gauge, 22 gauge, 23 gauge, 24 gauge, or 25 gauge. In some embodiments, the FNA sample from the patient can contain at least 400,000 TILs, *e.g.*, 400,000 TILs, 450,000 TILs, 500,000 TILs, 550,000 TILs, 600,000 TILs, 650,000 TILs, 700,000 TILs, 750,000 TILs, 800,000 TILs, 850,000 TILs, 900,000 TILs, 950,000 TILs, or more.

[00354] In some cases, the TILs described herein are obtained from a core biopsy sample. In some cases, the core biopsy sample is obtained or isolated from the patient using a surgical or

medical needle ranging from an 11 gauge needle to a 16 gauge needle. The needle can be 11 gauge, 12 gauge, 13 gauge, 14 gauge, 15 gauge, or 16 gauge. In some embodiments, the core biopsy sample from the patient can contain at least 400,000 TILs, *e.g.*, 400,000 TILs, 450,000 TILs, 500,000 TILs, 550,000 TILs, 600,000 TILs, 650,000 TILs, 700,000 TILs, 750,000 TILs, 800,000 TILs, 850,000 TILs, 900,000 TILs, 950,000 TILs, or more.

[00355] In general, the harvested cell suspension is called a “primary cell population” or a “freshly harvested” cell population

2. Pleural Effusion T-cells and TILs

[00356] In some embodiments, the sample is a pleural fluid sample. In some embodiments, the source of the T-cells or TILs for expansion according to the processes described herein is a pleural fluid sample. In some embodiments, the sample is a pleural effusion derived sample. In some embodiments, the source of the T-cells or TILs for expansion according to the processes described herein is a pleural effusion derived sample. See, for example, methods described in U.S. Patent Publication US 2014/0295426, incorporated herein by reference in its entirety for all purposes.

[00357] In some embodiments, any pleural fluid or pleural effusion suspected of and/or containing TILs can be employed. Such a sample may be derived from a primary or metastatic lung cancer, such as NSCLC or SCLC. In some embodiments, the sample may be secondary metastatic cancer cells which originated from another organ, *e.g.*, breast, ovary, colon or prostate. In some embodiments, the sample for use in the expansion methods described herein is a pleural exudate. In some embodiments, the sample for use in the expansion methods described herein is a pleural transudate. Other biological samples may include other serous fluids containing TILs, including, *e.g.*, ascites fluid from the abdomen or pancreatic cyst fluid. Ascites fluid and pleural fluids involve very similar chemical systems; both the abdomen and lung have mesothelial lines and fluid forms in the pleural space and abdominal spaces in the same matter in malignancies and such fluids in some embodiments contain TILs. In some embodiments, wherein the disclosure exemplifies pleural fluid, the same methods may be performed with similar results using ascites or other cyst fluids containing TILs.

[00358] In some embodiments, the pleural fluid is in unprocessed form, directly as removed from the patient. In some embodiments, the unprocessed pleural fluid is placed in a standard blood collection tube, such as an EDTA or Heparin tube, prior to the contacting step. In some embodiments, the unprocessed pleural fluid is placed in a standard CellSave® tube (Veridex) prior to the contacting step. In some embodiments, the sample is placed in the CellSave tube immediately after collection from the patient to avoid a decrease in the number of viable TILs. The number of viable TILs can decrease to a significant extent within 24 hours, if left in the untreated pleural fluid, even at 4°C. In some embodiments, the sample is placed in the appropriate collection tube within 1 hour, 5 hours, 10 hours, 15 hours, or up to 24 hours after removal from the patient. In some embodiments, the sample is placed in the appropriate collection tube within 1 hour, 5 hours, 10 hours, 15 hours, or up to 24 hours after removal from the patient at 4°C.

[00359] In some embodiments, the pleural fluid sample from the chosen subject may be diluted. In some embodiments, the dilution is 1:10 pleural fluid to diluent. In other embodiments, the dilution is 1:9 pleural fluid to diluent. In other embodiments, the dilution is 1:8 pleural fluid to diluent. In other embodiments, the dilution is 1:5 pleural fluid to diluent. In other embodiments, the dilution is 1:2 pleural fluid to diluent. In other embodiments, the dilution is 1:1 pleural fluid to diluent. In some embodiments, diluents include saline, phosphate buffered saline, another buffer or a physiologically acceptable diluent. In some embodiments, the sample is placed in the CellSave tube immediately after collection from the patient and dilution to avoid a decrease in the viable TILs, which may occur to a significant extent within 24-48 hours, if left in the untreated pleural fluid, even at 4°C. In some embodiments, the pleural fluid sample is placed in the appropriate collection tube within 1 hour, 5 hours, 10 hours, 15 hours, 24 hours, 36 hours, up to 48 hours after removal from the patient, and dilution. In some embodiments, the pleural fluid sample is placed in the appropriate collection tube within 1 hour, 5 hours, 10 hours, 15 hours, 24 hours, 36 hours, up to 48 hours after removal from the patient, and dilution at 4°C.

[00360] In still other embodiments, pleural fluid samples are concentrated by conventional means prior further processing steps. In some embodiments, this pre-treatment of the pleural fluid is preferable in circumstances in which the pleural fluid must be cryopreserved for shipment to a laboratory performing the method or for later analysis (*e.g.*, later than 24-48 hours post-collection). In some embodiments, the pleural fluid sample is prepared by centrifuging the

pleural fluid sample after its withdrawal from the subject and resuspending the centrifugate or pellet in buffer. In some embodiments, the pleural fluid sample is subjected to multiple centrifugations and resuspensions, before it is cryopreserved for transport or later analysis and/or processing.

[00361] In some embodiments, pleural fluid samples are concentrated prior to further processing steps by using a filtration method. In some embodiments, the pleural fluid sample used in the contacting step is prepared by filtering the fluid through a filter containing a known and essentially uniform pore size that allows for passage of the pleural fluid through the membrane but retains the tumor cells. In some embodiments, the diameter of the pores in the membrane may be at least 4 μM . In other embodiments the pore diameter may be 5 μM or more, and in other embodiment, any of 6, 7, 8, 9, or 10 μM . After filtration, the cells, including TILs, retained by the membrane may be rinsed off the membrane into a suitable physiologically acceptable buffer. Cells, including TILs, concentrated in this way may then be used in the contacting step of the method.

[00362] In some embodiments, pleural fluid sample (including, for example, the untreated pleural fluid), diluted pleural fluid, or the resuspended cell pellet, is contacted with a lytic reagent that differentially lyses non-nucleated red blood cells present in the sample. In some embodiments, this step is performed prior to further processing steps in circumstances in which the pleural fluid contains substantial numbers of RBCs. Suitable lysing reagents include a single lytic reagent or a lytic reagent and a quench reagent, or a lytic agent, a quench reagent and a fixation reagent. Suitable lytic systems are marketed commercially and include the BD Pharm Lyse™ system (Becton Dickinson). Other lytic systems include the Versalyse™ system, the FACSlyse™ system (Becton Dickinson), the Immunoprep™ system or Erythrolyse II system (Beckman Coulter, Inc.), or an ammonium chloride system. In some embodiments, the lytic reagent can vary with the primary requirements being efficient lysis of the red blood cells, and the conservation of the TILs and phenotypic properties of the TILs in the pleural fluid. In addition to employing a single reagent for lysis, the lytic systems useful in methods described herein can include a second reagent, *e.g.*, one that quenches or retards the effect of the lytic reagent during the remaining steps of the method, *e.g.*, Stabilyse™ reagent (Beckman Coulter, Inc.). A conventional fixation reagent may also be employed depending upon the choice of lytic reagents or the preferred implementation of the method.

[00363] In some embodiments, the pleural fluid sample, unprocessed, diluted or multiply centrifuged or processed as described herein above is cryopreserved at a temperature of about -140°C prior to being further processed and/or expanded as provided herein.

3. Tumor Fragmentation and /or Digest

[00364] As indicated above, in some embodiments, the TILs are derived from solid tumors. 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. In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the step of fragmentation is an *in vitro* or *ex-vivo* process. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the 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.

[00365] 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 mm³. In some embodiments, the tumor fragment is about 9 mm³. In some embodiments, the tumor fragment is about 10 mm³. In some embodiments, the tumor fragments are 1-4 mm x 1-4 mm x 1-4 mm. In some embodiments, the tumor fragments are 1 mm x 1 mm x 1 mm. In some embodiments, the tumor fragments are 2 mm x 2 mm x 2 mm. In some embodiments, the tumor fragments are 3 mm x 3 mm x 3 mm. In some embodiments, the tumor fragments are 4 mm x 4 mm x 4 mm.

[00366] In some embodiments, the tumors are fragmented in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of fatty tissue on each piece. In certain embodiments, the step of fragmentation of the tumor is an *in vitro* or *ex-vivo* method.

[00367] 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 mg/mL gentamicin, 30 U/mL DNase, and 1.0 mg/mL collagenase, followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). After placing the tumor in enzyme media, the tumor can be mechanically dissociated for approximately 1 minute. The solution can then be incubated for 30 minutes at 37 °C in 5% 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.

[00368] In some embodiments, the cell suspension prior to the first expansion step is called a “primary cell population” or a “freshly obtained” or “freshly isolated” cell population.

[00369] In some embodiments, a tumor lysate can be further obtained from the tumor digest through several freeze-thaw cycles or mass spectrometry procedures.

[00370] In some embodiments, the tumor fragments and/or tumor digest and/or tumor lysate can be optionally frozen and stored frozen prior to entry into the first expansion step, the TIL co-culture step, or the DC pulse step as described in further detail below.

[00371] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00372] In some embodiments, the enzyme mixture comprises collagenase. In some embodiments, the collagenase is collagenase IV. In some embodiments, the working stock for the collagenase is a 100 mg/mL 10X working stock.

[00373] In some embodiments, the enzyme mixture comprises DNase. In some embodiments, the working stock for the DNase is a 10,000IU/mL 10X working stock.

[00374] In some embodiments, the enzyme mixture comprises hyaluronidase. In some embodiments, the working stock for the hyaluronidase is a 10 mg/mL 10X working stock.

[00375] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 1000 IU/mL DNase, and 1 mg/mL hyaluronidase.

[00376] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 500 IU/mL DNase, and 1 mg/mL hyaluronidase.

[00377] 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 some embodiments, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients.

[00378] In some embodiments, the TILs are not obtained from tumor digests. In some embodiments, the solid tumor cores are not fragmented.

[00379] In some embodiments, obtaining the first population of TILs comprises a multilesional sampling method.

[00380] Tumor dissociating enzyme mixtures can include one or more dissociating (digesting) enzymes such as, but not limited to, collagenase (including any blend or type of collagenase), Accutase™, Accumax™, hyaluronidase, neutral protease (dispace), chymotrypsin, chymopapain, trypsin, caseinase, elastase, papain, protease type XIV (pronase), deoxyribonuclease I (DNase), trypsin inhibitor, any other dissociating or proteolytic enzyme, and any combination thereof.

[00381] In some embodiments, the dissociating enzymes are reconstituted from lyophilized enzymes. In some embodiments, lyophilized enzymes are reconstituted in an amount of sterile buffer such as Hank's balance salt solution (HBSS).

[00382] In some instances, collagenase (such as animal free- type 1 collagenase) is reconstituted in 10 mL of sterile HBSS or another buffer. The lyophilized stock enzyme may be at a concentration of 2892 PZ U/vial. In some embodiments, collagenase is reconstituted in 5 mL to 15 mL buffer. In some embodiment, after reconstitution the collagenase stock ranges from about 100 PZ U/mL-about 400 PZ U/mL, *e.g.*, about 100 PZ U/mL-about 400 PZ U/mL, about 100 PZ U/mL-about 350 PZ U/mL, about 100 PZ U/mL-about 300 PZ U/mL, about 150 PZ U/mL-about 400 PZ U/mL, about 100 PZ U/mL, about 150 PZ U/mL, about 200 PZ U/mL, about 210 PZ U/mL, about 220 PZ U/mL, about 230 PZ U/mL, about 240 PZ U/mL, about 250 PZ U/mL, about 260 PZ U/mL, about 270 PZ U/mL, about 280 PZ U/mL, about 289.2 PZ U/mL, about 300 PZ U/mL, about 350 PZ U/mL, or about 400 PZ U/mL.

[00383] In some embodiments neutral protease is reconstituted in 1 mL of sterile HBSS or another buffer. The lyophilized stock enzyme may be at a concentration of 175 DMC U/vial. In some embodiments, after reconstitution the neutral protease stock ranges from about 100 DMC/mL-about 400 DMC/mL, *e.g.*, about 100 DMC/mL-about 400 DMC/mL, about 100 DMC/mL-about 350 DMC/mL, about 100 DMC/mL-about 300 DMC/mL, about 150 DMC/mL-about 400 DMC/mL, about 100 DMC/mL, about 110 DMC/mL, about 120 DMC/mL, about 130 DMC/mL, about 140 DMC/mL, about 150 DMC/mL, about 160 DMC/mL, about 170 DMC/mL,

about 175 DMC/mL, about 180 DMC/mL, about 190 DMC/mL, about 200 DMC/mL, about 250 DMC/mL, about 300 DMC/mL, about 350 DMC/mL, or about 400 DMC/mL.

[00384] In some embodiments, DNase I is reconstituted in 1 mL of sterile HBSS or another buffer. The lyophilized stock enzyme was at a concentration of 4 KU/vial. In some embodiments, after reconstitution the DNase I stock ranges from about 1 KU/mL to 10 KU/mL, *e.g.*, about 1 KU/mL, about 2 KU/mL, about 3 KU/mL, about 4 KU/mL, about 5 KU/mL, about 6 KU/mL, about 7 KU/mL, about 8 KU/mL, about 9 KU/mL, or about 10 KU/mL.

[00385] In some embodiments, the stock of enzymes could change so verify the concentration of the lyophilized stock and amend the final amount of enzyme added to the digest cocktail accordingly

[00386] In some embodiments, the enzyme mixture includes about 10.2- μ L of neutral protease (0.36 DMC U/mL), 21.3- μ L of collagenase (1.2 PZ/mL) and 250- μ L of DNase I (200 U/mL) in about 4.7 mL of sterile HBSS.

4. Preparation of Crude Digest and Isolated Tumor Peptides

[00387] In some embodiments, a portion of tumor fragments is cryopreserved as a tumor cell suspension. In some embodiments, this suspension is thawed for use in later steps. In some embodiments, the thawed suspension is subjected to a dead cell removal kit before further use. In some embodiments, the thawed suspension is used without removing dead cells. In some embodiments, the tumor cell suspension is subjected to multiple freeze-thaw cycles. In different embodiments, the tumor cell suspension is subjected to 1, 2, 3, 4, 5 or 10 freeze-thaw cycles.

[00388] In other embodiments, the thawed suspension is further processed to produce tumor peptides, the process comprising: homogenizing the tumor into fine pieces, adding extract buffer at a ratio of about 50:1, dissolving the resultant protein pellet in a volume of 8M urea, 2M thioiurea and 400mM Ammonium biocarbonate with protease inhibitor, adding DTT, heating then cooling the solution, and trypsinizing the solution.

5. Mature Dendritic Cell Generation

[00389] In some embodiments, the methods disclosed herein comprise generation of mature dendritic cells (DCs) using the tumor digest or tumor lysate described herein. In some

embodiments, the DCs are derived from peripheral blood monocytes. Means of generating mature DCs from monocytes are well-known in the art. Briefly, peripheral-blood mononuclear cells (PBMCs) from a cancer patient apheresis or blood sample are incubated until monocytes adhere to a substrate. Monocytes are cultured in a cell culture medium containing GM-CSF and IL-4 for about 6 days to generate immature DCs. Immature DCs are then incubated with tumor digest or tumor lysate in a cell culture medium containing TNF α , IL-6, and IL-1 β to generate mature DCs. In some embodiments, the incubation lasts for 12 hours. In some embodiments, the incubation lasts for 16 hours. In some embodiments, the incubation lasts for 18 hours. In some embodiments, the incubation lasts for 24 hours. In some embodiments, the incubation lasts for 48 hours. In some embodiments, the incubation lasts for 72 hours. In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 10:1 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 5:1 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 3:1 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 2:1 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 1:1 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 1:2 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 1:3 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 1:5 (cell number). In some embodiments, the incubation comprises DC:tumor lysate at a ratio of about 1:10 (cell number).

6. Organoid/Tumoroid Generation

[00390] Tumors possess particular physical properties that are difficult to mimic using traditional cell culture models (see generally Dao et al., *Trends in Cancer*, 2022, 8:10, pages 870-880). As such, in one aspect, the methods disclosed herein include generation of organoids and/or tumoroids from the tumor digest/tumor lysate for the co-culture with TILs after the first expansion step in order to enrich tumor reactive TILs. In some embodiments, the organoid and/or tumoroid is cultured from freshly biopsied primary tissue. In some embodiments, the organoids and/or tumoroids are cultured from cryopreserved primary tissue. In some embodiments, the organoids and/or tumoroids are cultured from one or more tumor fragments or tumor digests. In some embodiments, the organoids and/or tumoroids are cultured from one or

more tumor fragments or tumor peptides. In some embodiments, the organoids and/or tumoroids are cultured from tumor-derived cells. In some embodiments, the organoids and/or tumoroids are cultured from a single donor. In some embodiments the organoids and/or tumoroids are cultured from more than one donor. In some embodiments, the organoids and/or tumoroids are cultured for use in an autologous therapy. In some embodiments, the organoids and/or tumoroids are cultured for use in an allogeneic therapy. In some embodiments, the organoids and/or tumoroids comprise a non-diseased tissue. In some embodiments, the organoids and/or tumoroids comprise an abnormal or cancerous tissue. In some embodiments, the tissue to become the organoids and/or tumoroids is cultured in with a liquid matrix and manipulated to polymerize, taking on an organ-like appearance.

[00391] In some embodiments, the organoids or tumoroids are cultured from a fine-needle aspirate. In some embodiments, FNA cells are cultured with Matrigel to promote organoid or tumoroid formation. In some embodiments, a stable cell line is generated from cells of the formed organoids or tumoroids. Additional experimental details regarding particular embodiments are described in Vilgelm, et al. (2020) *iScience*, 23(8), 101408, the content of which is hereby incorporated by reference in its entirety.

[00392] In some embodiments, organoids and/or tumoroids are useful for determining responses of the tumor from which they are derived to particular therapies. Additional experimental details regarding particular embodiments are described in Example 10 and in Dao et al., *Trends in Cancer*, 2022, 8:10, pages 870-880, the content of which is hereby incorporated by reference in its entirety.

B. First Expansion

[00393] In some embodiments, the methods disclosed herein provide for tumor-reactive TILs, which may provide additional therapeutic benefits over bystander TILs (*i.e.*, TILs that are competent to expand but do not react to cancerous tissues or cells). The dichotomy between tumor-reactive TILs and bystander TILs have been described in the art in at least the following, each of which is incorporated herein by reference: Simoni, Y., *et al.* (2018). *Nature*, 557(7706), 575-579; Meier, S. L., *et al.* (2022). *Nature Cancer*, 3(2), 143-155.

[00394] After dissection of tumor tissues and/or tumor fragments, 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 IL-2 is added at culture initiation along with the tumor digest and/or tumor fragments (*e.g.*, at Day 0). In some embodiments, the tumor and/or tumor fragments are incubated in a container with up to 60 fragments per container and with 6000 IU/mL of IL-2. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, first expansion occurs for a period of 1 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, first expansion occurs for a period of 1 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of 5 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of 5 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of about 6 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of about 6 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of about 7 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of about 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this first expansion occurs for a period of about 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells.

[00395] In some embodiments, a first expansion of TILs may be performed using processes, which can include those referred to as pre-REP or priming REP and which contain OKT-3, and feeder cells (*e.g.*, antigen-presenting feeder cells) from Day 0 and/or from culture initiation) as described below and herein, followed by a rapid second expansion (Step G, including processes referred to as rapid expansion protocol (REP) steps) as described below under Step G and herein, followed by optional cryopreservation. The TILs obtained from this process may be optionally

characterized for phenotypic characteristics and metabolic parameters as described herein. In some embodiments, the tumor fragment is between about 1 mm³ and 10 mm³.

[00396] In some embodiments, the first expansion culture medium is referred to as “CM”, an abbreviation for culture media. In some embodiments, CM for Step B consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin.

[00397] In some embodiments, there are less than or equal to 240 tumor fragments. In some embodiments, there are less than or equal to 240 tumor fragments placed in less than or equal to 4 containers. In some embodiments, the containers are GREX100 MCS flasks. In some embodiments, less than or equal to 60 tumor fragments are placed in 1 container. In some embodiments, each container comprises less than or equal to 500 mL of media per container. In some embodiments, the media comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media comprises antigen-presenting feeder cells (also referred to herein as “antigen-presenting cells”). In some embodiments, the media comprises 2.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media comprises OKT-3. In some embodiments, the media comprises 30 ng/mL of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng of OKT-3, and 2.5×10^8 antigen-presenting feeder cells. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5×10^8 antigen-presenting feeder cells per container.

[00398] After preparation of the tumor fragments, the resulting cells (*i.e.*, fragments which is a primary cell population) are cultured in media containing IL-2, antigen-presenting feeder cells and OKT-3 under conditions that favor the growth of TILs over tumor and other cells and which allow for TIL priming and accelerated growth from initiation of the culture on Day 0. In some embodiments, the tumor digests and/or tumor fragments are incubated in with 6000 IU/mL of IL-2, as well as antigen-presenting feeder cells and OKT-3. This primary cell population is cultured for a period of days, generally from 1 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, the growth media during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the growth media during the first expansion further comprises antigen-presenting feeder cells and OKT-3 as well. In some

embodiments, this primary cell population is cultured for a period of days, generally from 1 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, the growth media during the first expansion comprises IL-2 or a variant thereof, as well as antigen-presenting feeder cells and OKT-3. In some embodiments, the IL-2 is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of $20\text{-}30 \times 10^6$ 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 prepared as described in Example 4.

[00399] 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 6,000 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 some embodiments, the cell culture medium further comprises IL-2. In some embodiments, the first expansion cell culture medium comprises about 3,000 IU/mL of IL-2. In some embodiments, the first expansion cell culture medium further comprises IL-2. In some embodiments, the first cell culture medium comprises about 3,000 IU/mL of IL-2. In some embodiments, the first expansion cell culture medium comprises about 1,000 IU/mL, about 1,500 IU/mL, about 2,000 IU/mL, about 2,500 IU/mL, about 3,000 IU/mL, about 3,500 IU/mL, about 4,000 IU/mL, about 4,500 IU/mL, about 5,000 IU/mL, about 5,500 IU/mL, about 6,000 IU/mL, about 6,500 IU/mL, about 7,000 IU/mL, about 7,500 IU/mL, or about 8,000 IU/mL of IL-2. In some embodiments, the first expansion cell culture medium comprises between 1,000 and 2,000 IU/mL, between 2,000 and 3,000 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.

[00400] 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 first expansion cell culture medium comprises about 180 IU/mL of IL-15. In some embodiments, the first expansion cell culture medium further comprises IL-15. In some embodiments, the first expansion cell culture medium comprises about 180 IU/mL of IL-15.

[00401] 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 first expansion cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the first expansion cell culture medium comprises about 0.5 IU/mL of IL-21. In some embodiments, the cell culture medium further comprises IL-21. In some embodiments, the first expansion cell culture medium comprises about 1 IU/mL of IL-21.

[00402] In some embodiments, the first expansion cell culture medium comprises OKT-3 antibody. In some embodiments, the first expansion cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In some embodiments, the first expansion cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 µg/mL of OKT-3 antibody. In some embodiments, 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 comprises between 15 ng/mL and 30 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium comprises 30 ng/mL of OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab. *See*, for example, Table 1.

[00403] In some embodiments, the first expansion cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 µg/mL and 100 µg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 µg/mL and 40 µg/mL.

[00404] In some embodiments, in addition to one or more TNFRSF agonists, the first expansion cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist. In some embodiments, in addition to one or more TNFRSF agonists, the first expansion cell culture medium further comprises IL-2 at an initial concentration of about 6000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

[00405] 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, CM1 consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments, the CM is the CM1 described in the Examples. In some embodiments, the first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the first expansion culture medium or the initial cell culture medium or the first cell culture medium comprises IL-2, OKT-3 and antigen-presenting feeder cells (also referred to herein as feeder cells).

[00406] 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.

[00407] 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.

[00408] In some embodiments, the serum supplement or serum replacement includes, but is not limited to one or more of CTS™ OpTmizer T-Cell Expansion Serum Supplement, CTS™ Immune Cell Serum Replacement, one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more antibiotics, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline,

L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^+ , Al^{3+} , Ba^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Ge^{4+} , Se^{4+} , Br, T, Mn^{2+} , P, Si^{4+} , V^{5+} , Mo^{6+} , Ni^{2+} , Rb^+ , Sn^{2+} and Zr^{4+} . In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[00409] In some embodiments, the CTSTTMOpTmizerTM T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTSTTM OpTmizerTM T-cell Expansion Basal Medium, CTSTTM OpTmizerTM T-cell Expansion SFM, CTSTTM AIM-V Medium, CSTTM AIM-V SFM, LymphoONETM 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.

[00410] 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.

[00411] In some embodiments, the serum-free or defined medium is CTSTTM OpTmizerTM T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTSTTM OpTmizerTM is useful in the present invention. CTSTTM OpTmizerTM T-cell Expansion SFM is a combination of 1 L CTSTTM OpTmizerTM T-cell Expansion Basal Medium and 26 mL CTSTTM OpTmizerTM T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTSTTM OpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTSTTM OpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In

some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55μM.

[00412] In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1 L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 6000 IU/mL of IL-2. In

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

[00413] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (*i.e.*, GlutaMAX®) at a concentration of from about 0.1 mM to about 10mM, 0.5 mM to about 9 mM, 1 mM to about 8 mM, 2 mM to about 7 mM, 3 mM to about 6 mM, or 4 mM 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 2 mM.

[00414] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5 mM to about 150 mM, 10 mM to about 140 mM, 15 mM to about 130 mM, 20 mM to about 120 mM, 25 mM to about 110 mM, 30 mM to about 100 mM, 35 mM to about 95 mM, 40 mM to about 90 mM, 45 mM to about 85 mM, 50 mM to about 80 mM, 55 mM to about 75 mM, 60 mM to about 70 mM, or about 65 mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55 mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55 µM.

[00415] In some embodiments, the defined media described in International PCT Publication No. WO/1998/030679, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum- free culture. The

serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L- isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^+ , Al^{3+} , Ba^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Ge^{4+} , Se^{4+} , Br, T, Mn^{2+} , P, Si^{4+} , V^{5+} , Mo^{6+} , Ni^{2+} , Rb^+ , Sn^{2+} and Zr^{4+} . In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (α MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00416] 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.

[00417] 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 5. 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 5. 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 5.

TABLE 5. 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

[00418] In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μ M), 2-mercaptoethanol (final concentration of about 100 μ M).

[00419] In some embodiments, the defined media described in Smith, *et al.*, *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.

[00420] In some embodiments, 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 some embodiments, 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).

[00421] In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 1 to 11 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 2 to 11 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 3 to 11 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 4 to 11 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 5 to 11 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 6 to 11 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 7 to 11 days. In some embodiments, the first expansion (including processes such as those

[illegible]

[illegible]

pre-REP or priming REP) process is about 6 to 7 days. In some embodiments, the first expansion (including processes such as those sometimes referred to as the pre-REP or priming REP) process is about 7 days.

[00422] In some embodiments, the first TIL expansion can proceed for 1 days to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 1 days to 7 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 2 days to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 2 days to 7 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 3 days to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 3 days to 7 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 4 days to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 4 days to 7 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 5 days to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 5 days to 7 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 6 days to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 6 days to 7 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 7 to 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 8 days from when fragmentation occurs and/or when the first expansion step is initiated. In some embodiments, the first TIL expansion can proceed for 7 days from when fragmentation occurs and/or when the first expansion step is initiated.

[00423] In some embodiments, the first expansion of the TILs can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, or 11 days. In some embodiments,

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

[00424] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the priming first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first expansion. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the priming first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during the first expansion.

[00425] In some embodiments, the first expansion is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-10 or a

G-REX-100. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-10.

1. Feeder Cells and Antigen Presenting Cells

[00426] In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during days 4-8. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during days 4-7. In some embodiments, the priming first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the priming expansion at any time during days 5-8. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during days 5-7. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during days 6-8. In some embodiments, the priming first procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during days 6-7. In some embodiments, the first expansion procedures described herein (for

example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during day 7 or 8. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during day 6. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during day 7. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during day 8. In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion, but rather are added during the first expansion at any time during day 9.

[00427] In some embodiments, the first expansion procedures described herein (for example including expansion such as those referred to as pre-REP or priming REP) require feeder cells (also referred to herein as “antigen-presenting cells”) at the initiation of the TIL expansion and during the first expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, 2.5×10^8 feeder cells are used during the first expansion. In some embodiments, 2.5×10^8 feeder cells per container are used during the first expansion. In some embodiments, 2.5×10^8 feeder cells per GREX-10 are used during the first expansion. In some embodiments, 2.5×10^8 feeder cells per GREX-100 are used during the first expansion.

[00428] In general, the allogeneic 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.

[00429] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the first expansion.

[00430] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 have not increased from the initial viable cell number put into culture on day 0 of the first expansion. In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody and 3000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody and 6000 IU/mL IL-2.

[00431] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 have not increased from the initial viable cell number put into culture on day 0 of the first expansion. In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/mL OKT3 antibody and 1000-6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/mL OKT3 antibody and 2000-5000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/mL OKT3 antibody and 2000-4000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/mL OKT3 antibody and 2500-3500 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody and 6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 15 ng/mL OKT3 antibody and 3000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 15 ng/mL OKT3 antibody and 6000 IU/mL IL-2.

[00432] 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 some embodiments, 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 some embodiments, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to

300. In some embodiments, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

[00433] In some embodiments, the first expansion procedures described herein require a ratio of about 2.5×10^8 feeder cells to about 100×10^6 TILs. In other embodiments, the first expansion procedures described herein require a ratio of about 2.5×10^8 feeder cells to about 50×10^6 TILs. In yet other embodiments, the first expansion described herein require about 2.5×10^8 feeder cells to about 25×10^6 TILs. In yet other embodiments, the first expansion described herein require about 2.5×10^8 feeder cells. In yet other embodiments, the first expansion requires one-fourth, one-third, five-twelfths, or one-half of the number of feeder cells used in the rapid second expansion.

[00434] In some embodiments, the media in the first expansion comprises IL-2. In some embodiments, the media in the first expansion comprises 6000 IU/mL of IL-2. In some embodiments, the media in the first expansion comprises antigen-presenting feeder cells. In some embodiments, the media in the first expansion comprises 2.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media in the first expansion comprises OKT-3. In some embodiments, the media comprises 30 ng of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5×10^8 antigen-presenting feeder cells. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media comprises 500 mL of culture medium and 15 μ g of OKT-3 per 2.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media comprises 500 mL of culture medium and 15 μ g of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 500 mL of culture medium, 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5×10^8 antigen-presenting feeder cells. In some embodiments, the media comprises 500 mL of culture medium, 6000 IU/mL of IL-2, 15 μ g of OKT-3, and 2.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media comprises 500 mL of culture medium and 15 μ g of OKT-3 per 2.5×10^8 antigen-presenting feeder cells per container.

[00435] In some embodiments, the first expansion procedures described herein require an excess of feeder cells over TILs during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

[00436] In general, the allogeneic 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.

[00437] In some embodiments, artificial antigen presenting cells are used in the first expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines and Other Additives

[00438] 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.

[00439] Alternatively, using combinations of cytokines for the first expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is described in U.S. Patent Application Publication No. US 2017/0107490 A1, the disclosure of which is incorporated by reference herein. 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. *See*, for example, Table 2.

[0003] In some embodiments, Step B may also include the addition of OKT-3 antibody or muromonab to the culture media, as described elsewhere herein. In some embodiments, Step B may also include the addition of a 4-1BB agonist to the culture media, as described elsewhere herein. In some embodiments, Step B may also include the addition of an OX-40 agonist to the culture media, as described elsewhere herein. In addition, additives such as peroxisome proliferator-activated receptor gamma coactivator I-alpha agonists, including proliferator-activated receptor (PPAR)-gamma agonists such as a thiazolidinedione compound, may be used

in the culture media during Step B, as described in U.S. Patent Application Publication No. US 2019/0307796 A1, the disclosure of which is incorporated by reference herein.

C. Enriching Tumor Reactive TILs

[00440] In some embodiments, the methods disclosed herein comprise enriching the tumor reactive TILs, for example, after the first expansion step. In some embodiments, before the enriching step, residual tumor fragments are removed from the TILs after the first expansion step.

[00441] In some embodiments, the enriching step comprises: (a) co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate; (b) co-culture of TILs from the first expansion with mature dendritic cells (that previously were cultured with autologous tumor antigens—either in the form of a tumor digest/tumor lysate or isolated peptides); or (c) co-culture of the TILs from the first expansion with autologous tumoroids or organoids, such that the tumor reactive TIL population becomes enriched.

[00442] In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 1:10. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 1:5. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 1:3. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 1:2. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 1:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 2:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 3:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 5:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate at a TIL:tumor cell ratio of 10:1.

[00443] In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 1:10. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 1:5. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 1:3. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 1:2. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 1:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 2:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 3:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 5:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with mature DCs at a TIL:DC ratio of 10:1.

[00444] In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 1:10. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 1:5. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 1:3. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 1:2. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 1:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 2:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 3:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 5:1. In some embodiments, the enriching step comprises co-culture of TILs from the first expansion step with autologous tumoroids or organoids at a TIL:tumor cell ratio of 10:1.

[00445] In some embodiments, the enriching step takes place for about 12 hours, about 16 hours, about 18 hours, about 24 hours, about 36 hours, about 48 hours, about 72 hours, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, or about 10 days.

[00446] In some embodiments, tumor reactivity of the TILs after the enrichment step may be assessed by measuring one or more characteristics of the TILs. For example, one or more secreted factors in the TIL cell culture supernatants (e.g., presence or absence, concentration, specific activity) may be measured. In some embodiments, the measurable secreted factors are one or more cytokines. In some embodiments, the measurable secreted factor is IFN- γ .

D. Identifying A Plurality Of Tumor Reactive TILs

[00447] In some embodiments, the methods disclosed herein further comprise identifying a plurality of tumor reactive TILs, which may be collected and further expanded as disclosed herein.

[00448] In some embodiments, identifying the plurality of tumor reactive TILs may comprise determining whether a TIL exhibits an activation signal identifying the TIL as tumor reactive, such as a change in cell morphology (e.g., immunological synapse formation, cell shape, etc.), a change in cell surface expression of one or more proteins, a change in secretion level of one or more cytokines, a change in expression of an mRNA, etc.

[00449] In some embodiments, the activation signal may comprise increased and/or decreased cell surface expression of one or more proteins. In some embodiments, the one or more proteins comprise a T cell activation or exhaustion marker. In some embodiments, the T cell activation or exhaustion marker is selected from the group consisting of: CD3, CD4, CD8, PD-1, LAG3, Tim3, TIGIT, CD103, CD39, CD134, CD137, CD25, CD69, HLA-DR, CD107A, CD40L, Ki67, CD45RA, CCR7, KLRG1, and combinations thereof.

[00450] In some embodiments, the T cell activation or exhaustion marker is a protein on the cell surface. In some embodiments, determining whether a TIL exhibits an activation signal is performed by staining the population of TILs after the enrichment step with antibodies to the T cell activation or exhaustion marker. In some embodiments, the antibodies are polyclonal antibodies. In some embodiments, the antibodies are monoclonal antibodies.

[00451] In some embodiments, the activation signal may comprise a cell morphology. In some embodiments, the cell morphology is a flattened, rounded cell morphology. See Lin, *et al.*, *BMC Immunol.* **2015**, 16, 49, the content of which is hereby incorporated by reference in its entirety. In some embodiments, the activation signal is a concentration of mitochondrial mass in proximity to the cell membrane of the TIL. In some embodiments, the cell morphology is determined by imaging-based cell separation methods. In some embodiments, the imaging-based cell separation method uses a cell sorting system, such as the methods and cell sorting systems described in WO 2020037070 A1 and US 2021/0190669 A1, the contents of which are hereby incorporated by reference in their entireties.

[00452] In some embodiments, the methods disclosed herein further comprise collecting the plurality of tumor reactive TILs. In some embodiments, collecting the plurality of tumor reactive TILs comprises separating the plurality of tumor reactive TILs from non-tumor reactive TILs. In some embodiments, the collecting is performed using a cell sorting method. In some embodiments, the cell sorting method is a flow cytometry method, *e.g.*, flow activated cell sorting (FACS). In some embodiments, the gating is set up for each sort. In some embodiments, the gating is set-up for each sample of TILs. In some embodiments, the gating template is set-up from TILs every 10 days, 20 days, 30 days, 40 days, 50 days, or 60 days. In some embodiments, the gating template is set-up from TILs every 60 days. In some embodiments, the gating template is set-up for each sample of TILs every 10 days, 20 days, 30 days, 40 days, 50 days, or 60 days. In some embodiments, the gating template is set-up for each sample of PBMC's every 60 days. In some embodiments, the flow cytometry is performed using a SONY FX 500, Miltenyi Tyto or Miltenyi CliniMACS flow-activated cell sorter.

[00453] In some embodiments, the collecting is performed using an imaging-based cell sorting method. In some embodiments, the method further comprises (i) providing a population of cells comprising the cell, (ii) analyzing a subpopulation of the population of cells for a first time to detect a target cell, (iii) if a number of the target cell in the subpopulation is above a predetermined threshold number, collect the subpopulation, and (iv) analyzing the subpopulation for a second time. In some embodiments, the method further comprises, in (ii), capturing one or more images of each cell of the subpopulation. In some embodiments, the method further comprises capturing a single image of each cell from a single angle.

[00454] In some embodiments, the method further comprises: (a) transporting a cell through a flow channel; (b) capturing a plurality of images of the cell from a plurality of different angles as the cell is transported through the flow channel; and (c) analyzing the plurality of images using a deep learning algorithm to sort the cell. In some embodiments, the method further comprises rotating the cell as the cell is being transported through the flow channel. In some embodiments, the method further comprises applying a velocity gradient across the cell to rotate the cell. In some embodiments, the cell is flown in a first buffer at a first velocity, and wherein the applying the velocity gradient across the cell comprises co-flowing a second buffer at a second velocity. In some embodiments, an axis of the rotation of the cell and an additional axis of migration of the cell along the flow channel are different. In some embodiments, the axis of the rotation of the cell is perpendicular to the additional axis of the migration of the cell along the flow channel. In some embodiments, the method further comprises focusing the cell into a streamline at a height within the flow channel as the cell is being transported through the flow channel. In some embodiments, the focusing comprises subjecting the cell under an inertial lift force, wherein the inertial lift force is characterized by a Reynolds number of greater than 1. In some embodiments, the inertial lift force is characterized by a Reynolds number of at least 20. In some embodiments, the plurality of images is captured at a rate of about 10 frames per second to about 500,000 frames per second. In some embodiments, the plurality of angles extends around the cell or over a portion of the cell. In some embodiments, the plurality of images of the cell are captured from (1) a top side of the cell, (2) a bottom side of the cell, (3) a front side of the cell, (4) a rear side of the cell, (5) a left side of the cell, or (6) a right side of the cell. In some embodiments, the plurality of images of the cell are captured from at least two sides selected from the group consisting of: (1) a top side of the cell, (2) a bottom side of the cell, (3) a front side of the cell, (4) a rear side of the cell, (5) a left side of the cell, and (6) a right side of the cell. In some embodiments, the method further comprises sorting the cell based on the analyzed plurality of images, by directing the cell to a selected channel of a plurality of channels downstream of the flow channel. In some embodiments, the plurality of channels excluding the selected channel are closed prior to directing the cell to the selected channel.

[00455] In some embodiments, the plurality of channels excluding the selected channel are closed using pressure, an electric field, a magnetic field, or a combination thereof. In some

embodiments, the method further comprises validating the sorting of the cell using a light. In some embodiments, the validating comprises determining information associated with the cell using blockage or scattering of the light. In some embodiments, the information associated with the cell comprises a size, shape, density, texture, or speed of the cell. In some embodiments, the validating comprises (i) providing at least two light spots on the selected channel by directing at least two lights towards the selected channel, and (ii) determining a travel time of the cell between the at least two light spots, wherein the at least two light spots are spaced apart by about 10 micrometer to about 1,000 micrometer. In some embodiments, the light comprises a laser. In some embodiments, the sorting comprises (i) directing a first cell to a first channel of the plurality of channels and (ii) directing a second cell to a second channel of the plurality of channels, wherein the first cell and the second cell have or are suspected of having one or more different features.

[00456] In some embodiments, the method further comprises sorting a plurality of cells at a rate of at least 10 cells per second, wherein the plurality of cells comprises the cell. In some embodiments, the method further comprises sorting a plurality of cells comprising the cell using a classifier; and feeding data from the sorting back to the classifier in order to train the classifier for future sorting. In some embodiments, the classifier comprises a neural network. In some embodiments, the classifier is configured to perform classification of each of the plurality of cells, based on classification probabilities corresponding to a plurality of analyzed plurality of images of the plurality of cells.

[00457] In some embodiments, the method further comprises: (a) obtaining spatial information during motion of a plurality of cells relative to a patterned optical structure; (b) using the spatial information to identify the one or more target cells from the plurality of cells; and (c) based at least in part on the one or more target cells identified in (b), separating or isolating the one or more target cells from the plurality of cells at a rate of at least 10 cells per second. In some embodiments, (a) comprises: (i) directing light from a light source through the patterned optical structure, (ii) directing light from the patterned optical structure to the plurality of cells, and (iii) directing light from the plurality of cells to the detector. In some embodiments, (a) comprises: (i) directing light from a light source to the plurality of cells, (ii) directing light from the plurality of cells through the patterned optical structure, and (iii) directing light from the patterned optical structure to the detector. In some embodiments, the patterned optical structure

comprises a disordered patterned optical structure. In some embodiments, (c) comprises computationally reconstructing morphologies of the cells at least in part through combinatorial use of one or more temporal waveforms comprising one or more intensity distributions imparted by the patterned optical structure. In some embodiments, the target cell is a tumor reactive TIL. In some embodiments, (b) comprises applying one or more machine learning classifiers on compressed waveforms corresponding to the spatial information to identify the one or more target cells. In some embodiments, the one or more machine learning classifiers attain one or more of a sensitivity, a specificity, and an accuracy of at least 70%. In some embodiments, the one or more machine learning classifiers are selected from the group consisting of: support vector machines, random forest, artificial neural networks, convolutional neural networks, deep learning, ultra-deep learning, gradient boosting, AdaBoosting, decision trees, linear regression, and logistic regression. In some embodiments, the plurality of cells are processed without image reconstruction. In some embodiments, the detector comprises a single pixel detector. In some embodiments, the single pixel detector comprises a photomultiplier tube. In some embodiments, the method further comprises reconstructing one or more images of the plurality of cells. In some embodiments, the method further comprises reconstructing a plurality of images of the plurality of cells, each image of the plurality comprising a different wavelength or a range of wavelengths. In some embodiments, the one or more images are free of blur artifacts. In some embodiments, the plurality of cells move at a rate of at least 1 m/s relative to the patterned optical structure. In some embodiments, (c) comprises: (i) sorting the plurality of cells into one or more groups of sorted cells based on results of analyzing the plurality of cells; and (ii) collecting the one or more target cells from the one or more groups of sorted cells. In some embodiments, (c) comprises sorting the plurality of cells into one or more groups of sorted cells based on morphologies of the plurality of cells. In some embodiments, the sorting is achieved at a rate of at least 10 cells per second. In some embodiments, the method further comprises collecting one or more of the groups of sorted cells to generate an enriched cell mixture. In some embodiments, the one or more groups of sorted cells have a purity of at least 70%. In some embodiments, the method further comprises subjecting one or more cells of the one or more groups of sorted cells to one or more assays. In some embodiments, the one or more assays are selected from the group consisting of: lysis, nucleic acid extraction, nucleic acid amplification, nucleic acid sequencing, and protein sequencing. In some embodiments, the method further comprises, prior to (a),

subjecting the cells to hydrodynamic flow focusing. In some embodiments, the method further comprises collecting a partial transmissive speckle pattern of the plurality of cells as the plurality of cells move relative to the patterned optical structure. In some embodiments, the spatial information corresponds with characteristics, properties, or information pertaining to the plurality of cells. In some embodiments, the spatial information corresponds one-to-one with the characteristics, properties, or information pertaining to the plurality of cells. In some embodiments, the characteristics, properties, or information pertaining to the plurality of cells comprise one or more members selected from the group consisting of: metabolic states, proliferation states, differentiation states, maturity states, expression of marker proteins, expression of marker genes, morphology of cells, morphology of organelles, positioning of organelles, size or extent of organelles, morphology of cytoplasm, positioning of cytoplasm, size or extent of cytoplasm, morphology of nucleus, positioning of nucleus, size or extent of nucleus, morphology of mitochondria, positioning of mitochondria, size or extent of mitochondria, morphology of lysosome, positioning of lysosome, size or extent of lysosome, distribution of molecules inside cells, distribution of peptides, polypeptides, or proteins inside cells, distribution of nucleic acids inside cells, distribution of glycans or polysaccharides inside cells, and distribution of lipids inside cells.

[00458] In some embodiments, TILs are profiled before being subjected to an imaging-based cell sorting method. In other embodiments, TILs are directly subjected to an imaging-based cell sorting method without profiling.

E. Gene-Editing TILs

[00459] In some embodiments, the methods disclosed herein comprise gene-editing at least a portion of the TILs, for example, after the first expansion step, after the enriching step, after the collection step, or after the second expansion step. In some embodiments, the methods disclosed herein comprise gene-editing the second population of TILs after the first expansion step. In some embodiments, the methods disclosed herein comprise gene-editing the third population of TILs after the enriching step, wherein the enriching step comprises: (a) co-culture of TILs from the first expansion step with autologous tumor digest or tumor lysate; (b) co-culture of TILs from the first expansion with mature dendritic cells (that previously were cultured with autologous

tumor antigens—either in the form of a tumor digest/tumor lysate or isolated peptides); or (c) co-culture of the TILs from the first expansion with autologous tumoroids or organoids, such that the tumor reactive TIL population becomes enriched. In some embodiments, the methods disclosed herein comprise gene-editing the plurality of tumor reactive TILs after the plurality of tumor reactive TILs is separated from the non-tumor reactive TILs. In some embodiments, the methods disclosed herein comprise gene-editing the fourth population of TILs after the second expansion step.

[00460] As used herein, “gene-editing,” “gene editing,” and “genome editing” refer to a type of genetic modification in which DNA is permanently modified in the genome of a cell, *e.g.*, DNA is inserted, deleted, modified or replaced within the cell’s genome. In some embodiments, gene-editing causes the expression of a DNA sequence to be silenced (sometimes referred to as a gene knockout) or inhibited/reduced (sometimes referred to as a gene knockdown). In accordance with embodiments of the present invention, gene-editing technology is used to enhance the effectiveness of a therapeutic population of TILs. Exemplary gene-editing processes/methods of the present invention, as well as gene-edited TIL products can also be found in International Patent Application No. PCT/US22/14425, U.S. Provisional Application Nos. 63/304,498 and 63/242,373, all of which are incorporated herein by reference in their entireties for all related purposes.

[00461] In some embodiments of the present invention directed to methods for expanding TIL populations, the methods comprise one or more steps of introducing into at least a portion of the TILs nucleic acids, *e.g.*, mRNAs, for transient expression of an immunomodulatory protein, *e.g.*, an immunomodulatory fusion protein comprising an immunomodulatory protein fused to a membrane anchor, in order to produce modified TILs with (i) reduced dependence on cytokines in when expanded in culture and/or (ii) an enhanced therapeutic effect. As used herein, “transient gene-editing,” “transient gene editing,” “transient phenotypic alteration,” “transient phenotypic modification,” “temporary phenotypic alteration,” “temporary phenotypic modification,” “transient cellular change,” “transient cellular modification,” “temporary cellular alteration,” “temporary cellular modification,” “transient expression,” “transient alteration of expression,” “transient alteration of protein expression,” “transient modification,” “transitory phenotypic alteration,” “non-permanent phenotypic alteration,” “transiently modified,” “temporarily modified,” “non-permanently modified,” “transiently altered,” “temporarily

altered”, grammatical variations of any of the foregoing, and any expressions of similar meaning, refer to a type of cellular modification or phenotypic change in which nucleic acid (*e.g.*, mRNA) is introduced into a cell, such as transfer of nucleic acid into a cell by electroporation, calcium phosphate transfection, viral transduction, etc., and expressed in the cell (*e.g.*, expression of an immunomodulatory protein, such as an immunomodulatory fusion protein comprising an immunomodulatory protein fused to a membrane anchor) in order to effect a transient or non-permanent phenotypic change in the cell, such as the transient display of membrane-anchored immunomodulatory fusion protein on the cell surface. In accordance with embodiments of the present invention, transient phenotypic alteration technology is used to reduce dependence on cytokines in the expansion of TILs in culture and/or enhance the effectiveness of a therapeutic population of TILs.

[00462] In some embodiments, a microfluidic platform is used for intracellular delivery of nucleic acids encoding the immunomodulatory fusion proteins provided herein. In some embodiments, the microfluidic platform is a SQZ vector-free microfluidic platform. The SQZ platform is capable of delivering nucleic acids and proteins, to a variety of primary human cells, including T cells (Sharei *et al.* PNAS 2013, as well as Sharei *et al.* PLOS ONE 2015 and Greisbeck *et al.* J. Immunology vol. 195, 2015). In the SQZ platform, the cell membranes of the cells for modification (*e.g.*, TILs) are temporarily disrupted by microfluidic constriction, thereby allowing the delivery of nucleic acids encoding the immunomodulatory fusion proteins into the cells. Such methods as described in International Patent Application Publication Nos. WO 2013/059343A1, WO 2017/008063A1, or WO 2017/123663A1, or U.S. Patent Application Publication Nos. US 2014/0287509A1, US 2018/0201889A1, or US 2018/0245089A1 (incorporated herein by reference in their entireties) can be employed with the present invention for delivering nucleic acids encoding the subject immunomodulatory fusion proteins to a population of TILs. In some embodiments, the delivered nucleic acid allows for transient protein expression of the immunomodulatory fusion proteins in the modified TILs. In some embodiments, the SQZ platform is used for stable incorporation of the delivered nucleic acid encoding the immunomodulatory fusion protein into the TIL cell genome. Additional exemplary disclosures for the SQZ platform and its use can be found in International Patent Application Publication No. WO/2019/136456, which is incorporated herein by reference in its entirety for all purposes.

[00463] As discussed above, embodiments of the present invention provide tumor infiltrating lymphocytes (TILs) that have been genetically modified via gene-editing to enhance their therapeutic effect (*e.g.*, expression of an immunomodulatory fusion protein on its cell surface). Embodiments of the present invention embrace genetic editing through nucleotide insertion (RNA or DNA) into a population of TILs for both promotion of the expression of one or more proteins and inhibition of the expression of one or more proteins, as well as combinations thereof. Embodiments of the present invention also provide methods for expanding TILs into a therapeutic population, wherein the methods comprise gene-editing the TILs. There are several gene-editing technologies that may be used to genetically modify a population of TILs, which are suitable for use in accordance with the present invention.

[00464] In some embodiments, a method of genetically modifying a population of TILs includes the step of stable incorporation of genes for production of one or more proteins. In some embodiments, a method of genetically modifying a population of TILs includes the step of retroviral transduction. In some embodiments, a method of genetically modifying a population of TILs includes the step of lentiviral transduction. Lentiviral transduction systems are known in the art and are described, *e.g.*, in Levine, *et al.*, Proc. Nat'l Acad. Sci. 2006, 103, 17372-77; Zufferey, *et al.*, Nat. Biotechnol. 1997, 15, 871-75; Dull, *et al.*, J. Virology 1998, 72, 8463-71, and U.S. Patent No. 6,627,442, the disclosures of each of which are incorporated by reference herein. In some embodiments, a method of genetically modifying a population of TILs includes the step of gamma-retroviral transduction. Gamma-retroviral transduction systems are known in the art and are described, *e.g.*, Cepko and Pear, Cur. Prot. Mol. Biol. 1996, 9.9.1-9.9.16, the disclosure of which is incorporated by reference herein. In some embodiments, a method of genetically modifying a population of TILs includes the step of transposon-mediated gene transfer. Transposon-mediated gene transfer systems are known in the art and include systems wherein the transposase is provided as DNA expression vector or as an expressible RNA or a protein such that long-term expression of the transposase does not occur in the transgenic cells, for example, a transposase provided as an mRNA (*e.g.*, an mRNA comprising a cap and poly-A tail). Suitable transposon-mediated gene transfer systems, including the salmonid-type Tel-like transposase (SB or Sleeping Beauty transposase), such as SB10, SB11, and SB100x, and engineered enzymes with increased enzymatic activity, are described in, *e.g.*, Hackett, *et al.*,

Mol. Therapy **2010**, *18*, 674-83 and U.S. Patent No. 6,489,458, the disclosures of each of which are incorporated by reference herein.

[00465] In some embodiments, a method of genetically modifying a population of TILs includes the step of stable incorporation of genes for production or inhibition (*e.g.*, silencing) of one or more proteins. In some embodiments, a method of genetically modifying a population of TILs includes the step of electroporation. Electroporation methods are known in the art and are described, *e.g.*, in Tsong, *Biophys. J.* **1991**, *60*, 297-306, and U.S. Patent Application Publication No. 2014/0227237 A1, the disclosures of each of which are incorporated by reference herein. Other electroporation methods known in the art, such as those described in U.S. Patent Nos. 5,019,034; 5,128,257; 5,137,817; 5,173,158; 5,232,856; 5,273,525; 5,304,120; 5,318,514; 6,010,613 and 6,078,490, the disclosures of which are incorporated by reference herein, may be used. In some embodiments, the electroporation method is a sterile electroporation method. In some embodiments, the electroporation method is a pulsed electroporation method. In some embodiments, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein the sequence of at least three DC electrical pulses has one, two, or three of the following characteristics: (1) at least two of the at least three pulses differ from each other in pulse amplitude; (2) at least two of the at least three pulses differ from each other in pulse width; and (3) a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses. In some embodiments, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein at least two of the at least three pulses differ from each other in pulse amplitude. In some embodiments, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary

changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein at least two of the at least three pulses differ from each other in pulse width. In some embodiments, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses. In some embodiments, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to induce pore formation in the TILs, comprising the step of applying a sequence of at least three DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to TILs, wherein the sequence of at least three DC electrical pulses has one, two, or three of the following characteristics: (1) at least two of the at least three pulses differ from each other in pulse amplitude; (2) at least two of the at least three pulses differ from each other in pulse width; and (3) a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses, such that induced pores are sustained for a relatively long period of time, and such that viability of the TILs is maintained. In some embodiments, a method of genetically modifying a population of TILs includes the step of calcium phosphate transfection. Calcium phosphate transfection methods (calcium phosphate DNA precipitation, cell surface coating, and endocytosis) are known in the art and are described in Graham and van der Eb, *Virology* **1973**, 52, 456-467; Wigler, *et al.*, *Proc. Natl. Acad. Sci.* **1979**, 76, 1373-1376; and Chen and Okayarea, *Mol. Cell. Biol.* **1987**, 7, 2745-2752; and in U.S. Patent No. 5,593,875, the disclosures of each of which are incorporated by reference herein. In some embodiments, a method of genetically modifying a population of TILs includes the step of liposomal transfection. Liposomal transfection methods, such as methods that employ a 1:1 (w/w) liposome formulation of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*n,n,n*-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) in filtered water, are known in the art and are described in Rose, *et al.*, *Biotechniques* **1991**, 10, 520-525 and Felgner, *et al.*, *Proc. Natl. Acad.*

Sci. USA, **1987**, 84, 7413-7417 and in U.S. Patent Nos. 5,279,833; 5,908,635; 6,056,938; 6,110,490; 6,534,484; and 7,687,070, the disclosures of each of which are incorporated by reference herein. In some embodiments, a method of genetically modifying a population of TILs includes the step of transfection using methods described in U.S. Patent Nos. 5,766,902; 6,025,337; 6,410,517; 6,475,994; and 7,189,705; the disclosures of each of which are incorporated by reference herein.

[00466] According to some embodiments, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at one or more immune checkpoint genes. Such programmable nucleases enable precise genome editing by introducing breaks at specific genomic loci, *i.e.*, they rely on the recognition of a specific DNA sequence within the genome to target a nuclease domain to this location and mediate the generation of a double-strand break at the target sequence. A double-strand break in the DNA subsequently recruits endogenous repair machinery to the break site to mediate genome editing by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). Thus, the repair of the break can result in the introduction of insertion/deletion mutations that disrupt (*e.g.*, silence, repress, or enhance) the target gene product.

[00467] Major classes of nucleases that have been developed to enable site-specific genomic editing include zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and CRISPR-associated nucleases (*e.g.*, CRISPR/Cas9). These nuclease systems can be broadly classified into two categories based on their mode of DNA recognition: ZFNs and TALENs achieve specific DNA binding via protein-DNA interactions, whereas CRISPR systems, such as Cas9, are targeted to specific DNA sequences by a short RNA guide molecule that base-pairs directly with the target DNA and by protein-DNA interactions. *See, e.g.*, Cox *et al.*, *Nature Medicine*, 2015, Vol. 21, No. 2.

[00468] Non-limiting examples of gene-editing methods that may be used in accordance with TIL expansion methods of the present invention include CRISPR methods, TALE methods, and ZFN methods, embodiments of which are described in more detail below. According to some embodiments, a method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the

method further comprises gene-editing at least a portion of the TILs by one or more of a CRISPR method, a TALE method or a ZFN method, in order to generate TILs that can provide an enhanced therapeutic effect. According to some embodiments, gene-edited TILs can be evaluated for an improved therapeutic effect by comparing them to non-modified TILs *in vitro*, e.g., by evaluating *in vitro* effector function, cytokine profiles, etc. compared to unmodified TILs.

[00469] In some embodiments of the present invention, electroporation is used for delivery of a gene editing system, such as CRISPR, TALEN, and ZFN systems. In some embodiments of the present invention, the electroporation system is a flow electroporation system. An example of a suitable flow electroporation system suitable for use with some embodiments of the present invention is the commercially-available MaxCyte STX system. There are several alternative commercially-available electroporation instruments which may be suitable for use with the present invention, such as the AgilePulse system or ECM 830 available from BTX-Harvard Apparatus, Cellaxess Elektra (Celectricon), Nucleofector (Lonza/Amara), GenePulser MXcell (BIORAD), iPorator-96 (Primax) or siPORTer96 (Ambion). In some embodiments of the present invention, the electroporation system forms a closed, sterile system with the remainder of the TIL expansion method. In some embodiments of the present invention, the electroporation system is a pulsed electroporation system as described herein, and forms a closed, sterile system with the remainder of the TIL expansion method.

[00470] In some embodiments, a microfluidic platform is used for delivery of the gene editing system. In some embodiments, the microfluidic platform is a SQZ vector-free microfluidic platform.

a. CRISPR Methods

[00471] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a CRISPR method (e.g., CRISPR/Cas9 or CRISPR/Cpf1). According to particular embodiments, the use of a CRISPR method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface of, and optionally causes one or more immune checkpoint genes

to be silenced or reduced in, at least a portion of the therapeutic population of TILs. Alternatively, the use of a CRISPR method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface of, and optionally causes one or more immune checkpoint genes to be enhanced in, at least a portion of the therapeutic population of TILs. In some embodiments, the at least one immunomodulatory composition comprises an immunomodulatory agent fused to a membrane anchor (*e.g.*, a membrane anchored immunomodulatory fusion protein described herein). In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-2, IL-7, IL-10, IL-12, IL-15, IL-18, IL-21, and a CD40 agonist (*e.g.*, a CD40L or an agonistic CD40 binding domain). In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-2, IL-12, IL-15, IL-18, IL-21, and a CD40 agonist. In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-12, IL-15, IL-18, IL-21, and a CD40 agonist.

[00472] CRISPR stands for “Clustered Regularly Interspaced Short Palindromic Repeats.” A method of using a CRISPR system for gene editing is also referred to herein as a CRISPR method. CRISPR systems can be divided into two main classes, Class 1 and Class 2, which are further classified into different types and sub-types. The classification of the CRISPR systems is based on the effector Cas proteins that are capable of cleaving specific nucleic acids. In Class 1 CRISPR systems the effector module consists of a multi-protein complex, whereas Class 2 systems only use one effector protein. Class 1 CRISPR includes Types I, III, and IV and Class 2 CRISPR includes Types II, V, and VI. While any of these types of CRISPR systems may be used in accordance with the present invention, there are three types of CRISPR systems which incorporate RNAs and Cas proteins that are preferred for use in accordance with the present invention: Types I (exemplified by Cas3), II (exemplified by Cas9), and III (exemplified by Cas10). The Type II CRISPR is one of the most well-characterized systems.

[00473] CRISPR technology was adapted from the natural defense mechanisms of bacteria and archaea (the domain of single-celled microorganisms). These organisms use CRISPR-derived RNA and various Cas proteins, including Cas9, to foil attacks by viruses and other foreign bodies by chopping up and destroying the DNA of a foreign invader. A CRISPR is a specialized region of DNA with two distinct characteristics: the presence of nucleotide repeats and spacers. Repeated sequences of nucleotides are distributed throughout a CRISPR region with short segments of foreign DNA (spacers) interspersed among the repeated sequences. In

the type II CRISPR/Cas system, spacers are integrated within the CRISPR genomic loci and transcribed and processed into short CRISPR RNA (crRNA). These crRNAs anneal to transactivating crRNAs (tracrRNAs) and direct sequence-specific cleavage and silencing of pathogenic DNA by Cas proteins. Target recognition by the Cas9 protein requires a "seed" sequence within the crRNA and a conserved dinucleotide-containing protospacer adjacent motif (PAM) sequence upstream of the crRNA-binding region. The CRISPR/Cas system can thereby be retargeted to cleave virtually any DNA sequence by redesigning the crRNA. Thus, according to certain embodiments, Cas9 serves as an RNA-guided DNA endonuclease that cleaves DNA upon crRNA-tracrRNA recognition. The crRNA and tracrRNA in the native system can be simplified into a single guide RNA (sgRNA) of approximately 100 nucleotides for use in genetic engineering. The sgRNA is a synthetic RNA that includes a scaffold sequence necessary for Cas-binding and a user-defined approximately 17- to 20-nucleotide spacer that defines the genomic target to be modified. Thus, a user can change the genomic target of the Cas protein by changing the target sequence present in the sgRNA. The CRISPR/Cas system is directly portable to human cells by co-delivery of plasmids expressing the Cas9 endo-nuclease and the RNA components (*e.g.*, sgRNA). Different variants of Cas proteins may be used to reduce targeting limitations (*e.g.*, orthologs of Cas9, such as Cpf1).

[00474] According to some embodiments, an engineered, programmable, non-naturally occurring Type II CRISPR-Cas system comprises a Cas9 protein and at least one guide RNA that targets and hybridizes to a target sequence of a DNA molecule in a TIL, wherein the DNA molecule encodes and the TIL expresses at least one immune checkpoint molecule, and the Cas9 protein cleaves the DNA molecules, whereby expression of the at least one immune checkpoint molecule is altered; and, wherein the Cas9 protein and the guide RNA do not naturally occur together. According to some embodiments, the expression of two or more immune checkpoint molecules is altered. According to some embodiments, the guide RNA(s) comprise a guide sequence fused to a tracr sequence. For example, the guide RNA may comprise crRNA-tracrRNA or sgRNA. According to aspects of the present invention, the terms "guide RNA", "single guide RNA" and "synthetic guide RNA" may be used interchangeably and refer to the polynucleotide sequence comprising the guide sequence, which is the approximately 17-20 bp sequence within the guide RNA that specifies the target site.

[00475] Variants of Cas9 having improved on-target specificity compared to Cas9 may also be used in accordance with embodiments of the present invention. Such variants may be referred to as high-fidelity Cas-9s. According to some embodiments, a dual nickase approach may be utilized, wherein two nickases targeting opposite DNA strands generate a DSB within the target DNA (often referred to as a double nick or dual nickase CRISPR system). For example, this approach may involve the mutation of one of the two Cas9 nuclease domains, turning Cas9 from a nuclease into a nickase. Non-limiting examples of high-fidelity Cas9s include eSpCas9, SpCas9-HF1 and HypaCas9. Such variants may reduce or eliminate unwanted changes at non-target DNA sites. *See, e.g.,* Slaymaker IM, *et al. Science*. **2015** Dec 1, Kleinstiver BP, *et al. Nature*. **2016** Jan 6, and Ran *et al., Nat Protoc.* 2013 Nov; 8(11):2281-2308, the disclosures of which are incorporated by reference herein.

[00476] Additionally, according to particular embodiments, Cas9 scaffolds may be used that improve gene delivery of Cas9 into cells and improve on-target specificity, such as those disclosed in U.S. Patent Application Publication No. 2016/0102324, which is incorporated by reference herein. For example, Cas9 scaffolds may include a RuvC motif as defined by (D-[I/L]-G-X-X-S-X-G-W-A) and/or a HNH motif defined by (Y-X-X-D-H-X-X-P-X-S-X-X-X-D-X-S), where X represents any one of the 20 naturally occurring amino acids and [I/L] represents isoleucine or leucine. The HNH domain is responsible for nicking one strand of the target dsDNA and the RuvC domain is involved in cleavage of the other strand of the dsDNA. Thus, each of these domains nick a strand of the target DNA within the protospacer in the immediate vicinity of PAM, resulting in blunt cleavage of the DNA. These motifs may be combined with each other to create more compact and/or more specific Cas9 scaffolds. Further, the motifs may be used to create a split Cas9 protein (*i.e.*, a reduced or truncated form of a Cas9 protein or Cas9 variant that comprises either a RuvC domain or a HNH domain) that is divided into two separate RuvC and HNH domains, which can process the target DNA together or separately.

[00477] According to particular embodiments, a CRISPR method comprises silencing or reducing the expression of one or more immune checkpoint genes in TILs by introducing a Cas9 nuclease and a guide RNA (*e.g.*, crRNA-tracrRNA or sgRNA) containing a sequence of approximately 17-20 nucleotides specific to a target DNA sequence of the immune checkpoint gene(s). The guide RNA may be delivered as RNA or by transforming a plasmid with the guide RNA-coding sequence under a promoter. The CRISPR/Cas enzymes introduce a double-strand

break (DSB) at a specific location based on a sgRNA-defined target sequence. DSBs may be repaired in the cells by non-homologous end joining (NHEJ), a mechanism which frequently causes insertions or deletions (indels) in the DNA. Indels often lead to frameshifts, creating loss of function alleles; for example, by causing premature stop codons within the open reading frame (ORF) of the targeted gene. According to certain embodiments, the result is a loss-of-function mutation within the targeted immune checkpoint gene.

[00478] Alternatively, DSBs induced by CRISPR/Cas enzymes may be repaired by homology-directed repair (HDR) instead of NHEJ. While NHEJ-mediated DSB repair often disrupts the open reading frame of the gene, homology directed repair (HDR) can be used to generate specific nucleotide changes ranging from a single nucleotide change to large insertions. According to some embodiments, HDR is used for gene editing immune checkpoint genes by delivering a DNA repair template containing the desired sequence into the TILs with the sgRNA(s) and Cas9 or Cas9 nickase. The repair template preferably contains the desired edit as well as additional homologous sequence immediately upstream and downstream of the target gene (often referred to as left and right homology arms).

[00479] According to particular embodiments, an enzymatically inactive version of Cas9 (deadCas9 or dCas9) may be targeted to transcription start sites in order to repress transcription by blocking initiation. Thus, targeted immune checkpoint genes may be repressed without the use of a DSB. A dCas9 molecule retains the ability to bind to target DNA based on the sgRNA targeting sequence. According to some embodiments of the present invention, a CRISPR method comprises silencing or reducing the expression of one or more immune checkpoint genes by inhibiting or preventing transcription of the targeted gene(s). For example, a CRISPR method may comprise fusing a transcriptional repressor domain, such as a Kruppel-associated box (KRAB) domain, to an enzymatically inactive version of Cas9, thereby forming, *e.g.*, a dCas9-KRAB, that targets the immune checkpoint gene's transcription start site, leading to the inhibition or prevention of transcription of the gene. Preferably, the repressor domain is targeted to a window downstream from the transcription start site, *e.g.*, about 500 bp downstream. This approach, which may be referred to as CRISPR interference (CRISPRi), leads to robust gene knockdown via transcriptional reduction of the target RNA.

[00480] According to particular embodiments, an enzymatically inactive version of Cas9 (deadCas9 or dCas9) may be targeted to transcription start sites in order to activate transcription. This approach may be referred to as CRISPR activation (CRISPRa). According to some embodiments, a CRISPR method comprises increasing the expression of one or more immune checkpoint genes by activating transcription of the targeted gene(s). According to such embodiments, targeted immune checkpoint genes may be activated without the use of a DSB. A CRISPR method may comprise targeting transcriptional activation domains to the transcription start site; for example, by fusing a transcriptional activator, such as VP64, to dCas9, thereby forming, *e.g.*, a dCas9-VP64, that targets the immune checkpoint gene's transcription start site, leading to activation of transcription of the gene. Preferably, the activator domain is targeted to a window upstream from the transcription start site, *e.g.*, about 50-400 bp downstream

[00481] Additional embodiments of the present invention may utilize activation strategies that have been developed for potent activation of target genes in mammalian cells. Non-limiting examples include co-expression of epitope-tagged dCas9 and antibody-activator effector proteins (*e.g.*, the SunTag system), dCas9 fused to a plurality of different activation domains in series (*e.g.*, dCas9-VPR) or co-expression of dCas9-VP64 with a modified scaffold gRNA and additional RNA-binding helper activators (*e.g.*, SAM activators).

[00482] According to other embodiments, a CRISPR-mediated genome editing method referred to as CRISPR assisted rational protein engineering (CARPE) may be used in accordance with embodiments of the present invention, as disclosed in US Patent No. 9,982,278, which is incorporated by reference herein. CARPE involves the generation of "donor" and "destination" libraries that incorporate directed mutations from single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) editing cassettes directly into the genome. Construction of the donor library involves cotransforming rationally designed editing oligonucleotides into cells with a guide RNA (gRNA) that hybridizes to a target DNA sequence. The editing oligonucleotides are designed to couple deletion or mutation of a PAM with the mutation of one or more desired codons in the adjacent gene. This enables the entire donor library to be generated in a single transformation. The donor library is retrieved by amplification of the recombinant chromosomes, such as by a PCR reaction, using a synthetic feature from the editing oligonucleotide, namely, a second PAM deletion or mutation that is simultaneously incorporated at the 3' terminus of the gene. This covalently couples the codon target mutations directed to a

PAM deletion. The donor libraries are then co-transformed into cells with a destination gRNA vector to create a population of cells that express a rationally designed protein library.

[00483] According to other embodiments, methods for trackable, precision genome editing using a CRISPR-mediated system referred to as Genome Engineering by Trackable CRISPR Enriched Recombineering (GEn-TraCER) may be used in accordance with embodiments of the present invention, as disclosed in US Patent No. 9,982,278, which is incorporated by reference herein. The GEn-TraCER methods and vectors combine an editing cassette with a gene encoding gRNA on a single vector. The cassette contains a desired mutation and a PAM mutation. The vector, which may also encode Cas9, is introduced into a cell or population of cells. This activates expression of the CRISPR system in the cell or population of cells, causing the gRNA to recruit Cas9 to the target region, where a dsDNA break occurs, allowing integration of the PAM mutation.

[00484] Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a CRISPR method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGF β , PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, TET2, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, TOX, SOCS1, ANKRD11, and BCOR.

[00485] Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a CRISPR method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-18, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00486] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a CRISPR method, and which may be used in accordance with embodiments of the present invention, are described in U.S. Patent Nos. 8,697,359; 8,993,233; 8,795,965; 8,771,945; 8,889,356; 8,865,406; 8,999,641; 8,945,839; 8,932,814; 8,871,445; 8,906,616; and 8,895,308, which are incorporated by reference herein. Resources for carrying

out CRISPR methods, such as plasmids for expressing CRISPR/Cas9 and CRISPR/Cpf1, are commercially available from companies such as GenScript.

[00487] In some embodiments, genetic modifications of populations of TILs, as described herein, may be performed using the CRISPR/Cpf1 system as described in U.S. Patent No. US 9,790,490, the disclosure of which is incorporated by reference herein. The CRISPR/Cpf1 system is functionally distinct from the CRISPR-Cas9 system in that Cpf1-associated CRISPR arrays are processed into mature crRNAs without the need for an additional tracrRNA. The crRNAs used in the CRISPR/Cpf1 system have a spacer or guide sequence and a direct repeat sequence. The Cpf1p-crRNA complex that is formed using this method is sufficient by itself to cleave the target DNA.

b. TALE Methods

[00488] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in WO2018081473, WO2018129332, or WO2018182817, wherein the method further comprises gene-editing at least a portion of the TILs by a TALE method. According to particular embodiments, the use of a TALE method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface, and optionally causes expression of one or more immune checkpoint genes to be silenced or reduced, in at least a portion of the therapeutic population of TILs. Alternatively, the use of a TALE method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface, and optionally causes expression of one or more immune checkpoint genes to be enhanced, in at least a portion of the therapeutic population of TILs.

[00489] TALE stands for “Transcription Activator-Like Effector” proteins, which include TALENs (“Transcription Activator-Like Effector Nucleases”). A method of using a TALE system for gene editing may also be referred to herein as a TALE method. TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair. TALE specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues (RVDs). Modular TALE repeats are linked together to recognize contiguous DNA sequences. A specific RVD in the DNA-binding domain recognizes a

base in the target locus, providing a structural feature to assemble predictable DNA-binding domains. The DNA binding domains of a TALE are fused to the catalytic domain of a type IIS FokI endonuclease to make a targetable TALE nuclease. To induce site-specific mutation, two individual TALEN arms, separated by a 14-20 base pair spacer region, bring FokI monomers in close proximity to dimerize and produce a targeted double-strand break.

[00490] Several large, systematic studies utilizing various assembly methods have indicated that TALE repeats can be combined to recognize virtually any user-defined sequence. Strategies that enable the rapid assembly of custom TALE arrays include Golden Gate molecular cloning, high-throughput solid-phase assembly, and ligation-independent cloning techniques. Custom-designed TALE arrays are also commercially available through Collectis BioResearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA). Additionally web-based tools, such as TAL Effector-Nucleotide Target 2.0, are available that enable the design of custom TAL effector repeat arrays for desired targets and also provides predicted TAL effector binding sites. *See Doyle, et al., Nucleic Acids Research*, **2012**, Vol. 40, W117-W122. Examples of TALE and TALEN methods suitable for use in the present invention are described in U.S. Patent Application Publication Nos. US 2011/0201118 A1; US 2013/0117869 A1; US 2013/0315884 A1; US 2015/0203871 A1 and US 2016/0120906 A1, the disclosures of which are incorporated by reference herein.

[00491] According to some embodiments of the present invention, a TALE method comprises silencing or reducing the expression of one or more immune checkpoint genes by inhibiting or preventing transcription of the targeted gene(s). For example, a TALE method may include utilizing KRAB-TALEs, wherein the method comprises fusing a transcriptional Kruppel-associated box (KRAB) domain to a DNA binding domain that targets the gene's transcription start site, leading to the inhibition or prevention of transcription of the gene.

[00492] According to other embodiments, a TALE method comprises silencing or reducing the expression of one or more immune checkpoint genes by introducing mutations in the targeted gene(s). For example, a TALE method may include fusing a nuclease effector domain, such as FokI, to the TALE DNA binding domain, resulting in a TALEN. FokI is active as a dimer; hence, the method comprises constructing pairs of TALENs to position the FOKL nuclease domains to adjacent genomic target sites, where they introduce DNA double strand

breaks. A double strand break may be completed following correct positioning and dimerization of FokI. Once the double strand break is introduced, DNA repair can be achieved via two different mechanisms: the high-fidelity homologous recombination pair (HRR) (also known as homology-directed repair or HDR) or the error-prone non-homologous end joining (NHEJ). Repair of double strand breaks via NHEJ preferably results in DNA target site deletions, insertions or substitutions, *i.e.*, NHEJ typically leads to the introduction of small insertions and deletions at the site of the break, often inducing frameshifts that knockout gene function. According to particular embodiments, the TALEN pairs are targeted to the most 5' exons of the genes, promoting early frame shift mutations or premature stop codons. The genetic mutation(s) introduced by TALEN are preferably permanent. Thus, according to some embodiments, the method comprises silencing or reducing expression of an immune checkpoint gene by utilizing dimerized TALENs to induce a site-specific double strand break that is repaired via error-prone NHEJ, leading to one or more mutations in the targeted immune checkpoint gene.

[00493] According to additional embodiments, TALENs are utilized to introduce genetic alterations via HRR, such as non-random point mutations, targeted deletion, or addition of DNA fragments. The introduction of DNA double strand breaks enables gene editing via homologous recombination in the presence of suitable donor DNA. According to some embodiments, the method comprises co-delivering dimerized TALENs and a donor plasmid bearing locus-specific homology arms to induce a site-specific double strand break and integrate one or more transgenes into the DNA.

[00494] According to other embodiments, a TALEN that is a hybrid protein derived from FokI and AvrXa7, as disclosed in U.S. Patent Publication No. 2011/0201118, may be used in accordance with embodiments of the present invention. This TALEN retains recognition specificity for target nucleotides of AvrXa7 and the double-stranded DNA cleaving activity of FokI. The same methods can be used to prepare other TALEN having different recognition specificity. For example, compact TALENs may be generated by engineering a core TALE scaffold having different sets of RVDs to change the DNA binding specificity and target a specific single dsDNA target sequence. *See* U.S. Patent Publication No. 2013/0117869. A selection of catalytic domains can be attached to the scaffold to effect DNA processing, which may be engineered to ensure that the catalytic domain is capable of processing DNA near the single dsDNA target sequence when fused to the core TALE scaffold. A peptide linker may also

be engineered to fuse the catalytic domain to the scaffold to create a compact TALEN made of a single polypeptide chain that does not require dimerization to target a specific single dsDNA sequence. A core TALE scaffold may also be modified by fusing a catalytic domain, which may be a TAL monomer, to its N-terminus, allowing for the possibility that this catalytic domain might interact with another catalytic domain fused to another TAL monomer, thereby creating a catalytic entity likely to process DNA in the proximity of the target sequences. *See* U.S. Patent Publication No. 2015/0203871. This architecture allows only one DNA strand to be targeted, which is not an option for classical TALEN architectures.

[00495] According to some embodiments of the present invention, conventional RVDs may be used to create TALENs that are capable of significantly reducing gene expression. In some embodiments, four RVDs, NI, HD, NN, and NG, are used to target adenine, cytosine, guanine, and thymine, respectively. These conventional RVDs can be used to, for instance, create TALENs targeting the PD-1 gene. Examples of TALENs using conventional RVDs include the T3v1 and T1 TALENs disclosed in Gautron *et al.*, *Molecular Therapy: Nucleic Acids* Dec. 2017, Vol. 9:312-321 (Gautron), which is incorporated by reference herein. The T3v1 and T1 TALENs target the second exon of the *PDCD1* locus where the PD-L1 binding site is located and are able to considerably reduce PD-1 production. In some embodiments, the T1 TALEN does so by using target SEQ ID NO:256 and the T3v1 TALEN does so by using target SEQ ID NO:257.

[00496] According to other embodiments, TALENs are modified using non-conventional RVDs to improve their activity and specificity for a target gene, such as disclosed in Gautron. Naturally occurring RVDs only cover a small fraction of the potential diversity repertoire for the hypervariable amino acid locations. Non-conventional RVDs provide an alternative to natural RVDs and have novel intrinsic targeting specificity features that can be used to exclude the targeting of off-site targets (sequences within the genome that contain a few mismatches relative to the targeted sequence) by TALEN. Non-conventional RVDs may be identified by generating and screening collections of TALEN containing alternative combinations of amino acids at the two hypervariable amino acid locations at defined positions of an array as disclosed in Juillerat, *et al.*, *Scientific Reports* 5, Article Number 8150 (2015), which is incorporated by reference herein. Next, non-conventional RVDs may be selected that discriminate between the nucleotides present at the position of mismatches, which can prevent TALEN activity at off-site sequences

while still allowing appropriate processing of the target location. The selected non-conventional RVDs may then be used to replace the conventional RVDs in a TALEN. Examples of TALENs where conventional RVDs have been replaced by non-conventional RVDs include the T3v2 and T3v3 PD-1 TALENs produced by Gautron. These TALENs had increased specificity when compared to TALENs using conventional RVDs.

[00497] According to additional embodiments, TALEN may be utilized to introduce genetic alterations to silence or reduce the expression of two genes. For instance, two separate TALEN may be generated to target two different genes and then used together. The molecular events generated by the two TALEN at their respective loci and potential off-target sites may be characterized by high-throughput DNA sequencing. This enables the analysis of off-target sites and identification of the sites that might result from the use of both TALEN. Based on this information, appropriate conventional and non-conventional RVDs may be selected to engineer TALEN that have increased specificity and activity even when used together. For example, Gautron discloses the combined use of T3v4 PD-1 and TRAC TALEN to produce double knockout CAR T cells, which maintained a potent *in vitro* anti-tumor function.

[00498] In some embodiments, the method of Gautron or other methods described herein may be employed to genetically-edit TILs, which may then be expanded by any of the procedures described herein.

[00499] According to other embodiments, TALENs may be specifically designed, which allows higher rates of DSB events within the target cell(s) that are able to target a specific selection of genes. *See* U.S. Patent Publication No. 2013/0315884. The use of such rare cutting endonucleases increases the chances of obtaining double inactivation of target genes in transfected cells, allowing for the production of engineered cells, such as T-cells. Further, additional catalytic domains can be introduced with the TALEN to increase mutagenesis and enhance target gene inactivation. The TALENs described in U.S. Patent Publication No. 2013/0315884 were successfully used to engineer T-cells to make them suitable for immunotherapy. TALENs may also be used to inactivate various immune checkpoint genes in T-cells, including the inactivation of at least two genes in a single T-cell. *See* U.S. Patent Publication No. 2016/0120906. Additionally, TALENs may be used to inactivate genes encoding targets for immunosuppressive agents and T-cell receptors, as disclosed in U.S. Patent

Publication No. 2018/0021379, which is incorporated by reference herein. Further, TALENs may be used to inhibit the expression of beta 2-microglobulin (B2M) and/or class II major histocompatibility complex transactivator (CIITA), as disclosed in U.S. Patent Publication No. 2019/0010514, which is incorporated by reference herein.

[00500] Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a TALE method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGF β , PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, TET2, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, **TOX**, **SOCS1**, ANKRD11, and BCOR.

c. Zinc Finger Methods

[00501] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a zinc finger or zinc finger nuclease method. According to particular embodiments, the use of a zinc finger method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface, and optionally causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a zinc finger method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface, and optionally causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

[00502] An individual zinc finger contains approximately 30 amino acids in a conserved $\beta\beta\alpha$ configuration. Several amino acids on the surface of the α -helix typically contact 3 bp in the major groove of DNA, with varying levels of selectivity. Zinc fingers have two protein domains. The first domain is the DNA binding domain, which includes eukaryotic transcription factors and

contain the zinc finger. The second domain is the nuclease domain, which includes the FokI restriction enzyme and is responsible for the catalytic cleavage of DNA.

[00503] The DNA-binding domains of individual ZFNs typically contain between three and six individual zinc finger repeats and can each recognize between 9 and 18 base pairs. If the zinc finger domains are specific for their intended target site then even a pair of 3-finger ZFNs that recognize a total of 18 base pairs can, in theory, target a single locus in a mammalian genome. One method to generate new zinc-finger arrays is to combine smaller zinc-finger "modules" of known specificity. The most common modular assembly process involves combining three separate zinc fingers that can each recognize a 3 base pair DNA sequence to generate a 3-finger array that can recognize a 9 base pair target site. Alternatively, selection-based approaches, such as oligomerized pool engineering (OPEN) can be used to select for new zinc-finger arrays from randomized libraries that take into consideration context-dependent interactions between neighboring fingers. Engineered zinc fingers are available commercially; Sangamo Biosciences (Richmond, CA, USA) has developed a propriety platform (CompoZr®) for zinc-finger construction in partnership with Sigma-Aldrich (St. Louis, MO, USA).

[00504] Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a zinc finger method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, **TOX**, **SOCS1**, ANKRD11, and BCOR.

[00505] Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a zinc finger method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-18, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00506] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a zinc finger method, which may be used in accordance with embodiments of the present invention, are described in U.S. Patent Nos. 6,534,261, 6,607,882,

6,746,838, 6,794,136, 6,824,978, 6,866,997, 6,933,113, 6,979,539, 7,013,219, 7,030,215, 7,220,719, 7,241,573, 7,241,574, 7,585,849, 7,595,376, 6,903,185, and 6,479,626, which are incorporated by reference herein.

[00507] Other examples of systems, methods, and compositions for altering the expression of a target gene sequence by a zinc finger method, which may be used in accordance with embodiments of the present invention, are described in Beane, *et al.*, *Mol. Therapy*, **2015**, 23 1380-1390, the disclosure of which is incorporated by reference herein.

d. Cas-CLOVER Methods

[00508] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a Cas-CLOVER method. According to particular embodiments, the use of a Cas-CLOVER method during the TIL expansion process causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a Cas-CLOVER method during the TIL expansion process causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

[00509] Cas-CLOVER is a dimeric, high-fidelity site-specific nuclease (SSN) that consists of a fusion of catalytically dead SpCas9 (dCas9) with the nuclease domain from a Clostridium Clo051 type II restriction endonuclease (Madison, *et al.*, “Cas-CLOVER is a novel high-fidelity nuclease for safe and robust generation of T SCM-enriched allogeneic CAR-T cells,” *Molecular Therapy - Nucleic Acids*, 2022). This yields a nuclease whose activity is predicated upon the dimerization of the Clo051 nuclease domain, enabled by RNA-guided recognition of two adjacent 20-nt target sequences. Unlike a paired nickase approach, *e.g.*, when using the Cas9-D10A mutant, monomeric Cas-CLOVER does not introduce a nick or a DSB. Cas-CLOVER has been shown to have low off-target nuclease activity.

[00510] Exemplary Cas-CLOVER systems include those described in WO2019/126578, the contents of which are incorporated herein by reference in their entirety. In embodiments, the

Cas-CLOVER system comprises a fusion protein comprising, consisting essentially of, or consisting of a DNA localization component and an effector molecule.

[00511] DNA localization components

[00512] In embodiments, the DNA localization components are capable of binding a specific DNA sequence. In embodiments, the DNA localization component is selected from, for example, a DNA-binding oligonucleotide, a DNA-binding protein, a DNA binding protein complex, and combinations thereof. Other suitable DNA binding components will be recognized by one of ordinary skill in the art.

[00513] In embodiments, the DNA localization components comprise an oligonucleotide directed to a specific locus or loci in the genome. The oligonucleotide may be selected from DNA, RNA, DNA/RNA hybrids, and combinations thereof.

[00514] In embodiments, the DNA localization components comprise a nucleotide binding protein or protein complex that binds an oligonucleotide when bound to a target DNA. The protein or protein complex may be capable of recognizing a feature selected from RNA-DNA heteroduplexes, R-loops, or combinations thereof. In embodiments, the DNA localization component comprises a protein or protein complex capable of recognizing an R-loop selected from Cas9, Cascade complex, RecA, RNase H, RNA polymerase, DNA polymerase, or a combination thereof. In embodiments, the DNA localization component comprises an engineered protein capable of binding to target DNA. In embodiments, the DNA localization component comprises a protein capable of binding a DNA sequence selected from meganuclease, zinc finger array, transcription activator-like (TAL) array, and combinations thereof. In embodiments, the DNA localization component comprises a protein that contains a naturally occurring DNA binding domain. In embodiments, the DNA localization component comprises a bZIP domain, a Helix-loop-helix, a Helix-turn-helix, a HMG-box, a Leucine zipper, a Zinc finger, or a combination thereof. In embodiments, the DNA localization component comprises an oligonucleotide directed to a specific locus in the genome. Exemplary oligonucleotides include, but are not limited to, DNA, RNA, DNA/RNA hybrids, and any combination thereof. In embodiments, the DNA localization component comprises a protein or a protein complex capable of recognizing a feature selected from RNA-DNA heteroduplexes, R-loops, and any combination thereof. Exemplary proteins or protein complexes capable of

recognizing an R-loop include, but are not limited to, Cas9, Cascade complex, RecA, RNase H, RNA polymerase, DNA polymerase, and any combination thereof. In embodiments, the protein or protein complex capable of recognizing an R-loop comprises Cas9. In embodiments, the DNA localization component comprises a protein capable of binding a DNA sequence selected from meganuclease, Zinc Finger array, TAL array, and any combination thereof. In embodiments, the DNA localization component comprises an oligonucleotide directed to a target location in a genome and a protein capable of binding to a target DNA sequence.

[00515] In embodiments, the DNA localization components comprise, consist essentially of, or consist of, at least one guide RNA (gRNA). In embodiments, the DNA localization components comprise, consist essentially of, or consist of, two gRNAs, wherein a first gRNA specifically binds to a first strand of a double-stranded DNA target sequence and a second gRNA specifically binds to a second strand of the double-stranded DNA target sequence. Alternatively, in embodiments, DNA localization components comprise, consist essentially of, or consist of, a DNA binding domain of a transcription activator-like effector nuclease (TALEN, also referred to as a TAL protein). In embodiments DNA localization components comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from *Xanthomonas* or *Ralstonia*.

[00516] Effector molecules

[00517] In embodiments, effector molecules are capable of a predetermined effect at a specific locus in the genome. Exemplary effector molecules, but are not limited to, a transcription factor (activator or repressor), chromatin remodeling factor, nuclease, exonuclease, endonuclease, transposase, methyltransferase, demethylase, acetyltransferase, deacetylase, kinase, phosphatase, integrase, recombinase, ligase, topoisomerase, gyrase, helicase, fluorophore, or any combination thereof.

[00518] In embodiments, effector molecules comprise a transposase. In embodiments, effector molecules comprise a PB transposase (PBase). In embodiments, effector molecules comprise a nuclease. Non-limiting examples of nucleases include restriction endonucleases, homing endonucleases, S1 nuclease, mung bean nuclease, pancreatic DNase I, micrococcal nuclease, yeast HO endonuclease, or any combination thereof. In certain embodiments, the effector molecule comprises a restriction endonuclease. In certain embodiments, the effector molecule

comprises a Type IIS restriction endonuclease. In embodiments, effector molecules comprise an endonuclease. Non-limiting examples of the endonuclease include AciI, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, MboII, MyII, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I and Clo051. In embodiments, the effector molecule comprises BmrI, BfiI, or Clo051.

[00519] In embodiments, effector molecules comprise, consist essentially of, or consist of, a homodimer or a heterodimer. In embodiments, effector molecules comprise, consist essentially of, or consist of, a nuclease, optionally an endonuclease. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a Cas9, a Cas9 nuclease domain or a fragment thereof. In embodiments, the Cas9 is a catalytically inactive or “inactivated” Cas9 (dCas9 (SEQ ID NO: 302 and 303 of WO2019/126578)). In embodiments, the Cas9 is a catalytically inactive or “inactivated” nuclease domain of Cas9. In embodiments, the dCas9 is encoded by a shorter sequence that is derived from a full length, catalytically inactivated, Cas9, referred to herein as a “small” dCas9 or dSaCas9 (SEQ ID NO: 23 of WO2019/126578).

[00520] In embodiments of the fusion protein, the effector molecule comprises, consists essentially of, or consists of a homodimer or a heterodimer of one or more Type II nucleases. In embodiments of the fusion protein, the effector molecule comprises, consists essentially of, or consists of a homodimer or a heterodimer of a Type II nuclease. In embodiments, the Type II nuclease comprises one or more of AciI, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, MboII, MyII, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I or Clo051.

[00521] In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, Clo051, BfiI or BmrI. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a Cas9, a Cas9

nuclease domain or a fragment thereof that forms a heterodimer with Clo051, BfiI or BmrI. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a catalytically-inactive form of Cas9 (*e.g.* dCas9 or dSaCas9) or a fragment thereof that forms a heterodimer with Clo051. An exemplary Clo051 nuclease domain may comprise, consist essentially of or consist of, the amino acid sequence of:

EGIKSNISLLKDELRGQISHISHEYLSLIDLAFDSKQNRLFEMKVLELLVNEYGFKGRH
LGGSRKPDGIVYSTTLEDNFGIIVDTKAYSEGYSLPISQADEMERYVRENSNRDEEVN
PNKWWENFSEEVKKYYFVFISGSFKGKFEEQLRRLSMTTGVNGSAVNVNLLLGAE
KIRSGEMTIEELERAMFNNSEFILKY (SEQ ID NO:238).

[00522] In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from *Xanthomonas* or *Ralstonia*. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from *Xanthomonas* or *Ralstonia* that forms a homodimer or a heterodimer with Clo051, BfiI or BmrI. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from *Xanthomonas* or *Ralstonia* that forms a homodimer or a heterodimer with Clo051.

[00523] Linkages

[00524] In embodiments, the fusion protein comprises, consists essentially of, or consists of, a DNA localization component and an effector molecule. In embodiments, the nucleic acid sequences encoding one or more components of the fusion protein can be operably linked, for example, in an expression vector. In embodiments, the fusion proteins are chimeric proteins. In embodiments, the fusion proteins are encoded by one or more recombinant nucleic acid sequences. In embodiments, the fusion proteins also include a linker region to operatively-link two components of the fusion protein. For example, in embodiments, the fusion protein comprises, consists essentially of, or consists, of a DNA localization component and an effector molecule, operatively-linked by a linker region. In embodiments, the DNA localization

component, the linker region, and the effector molecule can be encoded by one or more nucleic acid sequences inserted into an expression cassette and/or expression vector such that translation of the nucleic acid sequence results in the fusion protein. In embodiments, the fusion protein can comprise a non-covalent linkage between the DNA localization component and the effector molecule. The non-covalent linkage can comprise an antibody, an antibody fragment, an antibody mimetic, or a scaffold protein.

[00525] Fusion proteins

[00526] In embodiments, the DNA localization component comprises, consists essentially of or consists of, at least one gRNA, and the effector molecule comprises, consists essentially of, or consists of a Cas9, a Cas9 nuclease domain, or a fragment thereof. In embodiments, the DNA localization component comprises, consists essentially of, or consists of, at least one gRNA, and the effector molecule comprises, consists essentially of, or consists of an inactivated Cas9 (dCas9) or an inactivated nuclease domain. In embodiments, the DNA localization component comprises, consists essentially of, or consists of, at least one gRNA, and the effector molecule comprises, consists essentially of, or consists of an inactivated small Cas9 (dSaCas9). In embodiments, the effector molecule comprises, consists essentially of, or consists of a Cas9, dCas9, dSaCas9, or nuclease domain thereof, and a second endonuclease. The second endonuclease can comprise, consist essentially of, or consist of a Type IIS endonuclease, including, but not limited to, one or more of AciI, MnlI, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, MboII, MylI, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I, FokI or Clo051.

[00527] In embodiments of the fusion proteins, the DNA localization component comprises, consists essentially of, or consists of, a DNA-binding domain of a transcription activator-like effector nuclease (TALEN, also referred to as a TAL protein), and the effector molecule comprises, consists essentially of, or consists of, an endonuclease. In embodiments of the fusion proteins of the disclosure, the DNA localization component comprises, consists essentially of, or consists of, a DNA-binding domain of a TALEN, or TAL protein, derived from *Xanthomonas* or *Ralstonia*, and the effector molecule comprises, consists essentially of, or consists of, a Type IIS endonuclease, including, but not limited to, one or more of AciI, MnlI, AlwI, BbvI, BccI,

BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, MboII, MyII, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I or Clo051.

[00528] In certain embodiments, an exemplary dCas9-Clo051 fusion protein may comprise, consist essentially of or consist of the amino acid sequence of SEQ ID NO: 305 or 307 of WO2019/126578 or the nucleic acid sequence of SEQ ID NO: 306 or 308 of WO2019/126578.

[00529] Constructs

[00530] In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a dCas9 and Clo051. In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a dSaCas9 and Clo051. In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a Xanthomonas-TALE and Clo051. In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a Ralstonia-TALE and Clo051. In embodiments, the fusion protein comprises dCas9-Clo051, dSaCas9-Clo051, Xanthomonas-TALE-Clo051, or Ralstonia-TALE-Clo051. In embodiments, a vector encoding the fusion protein comprises Csy4-T2A-Clo051-G4Slinker-dCas9 (*Streptococcus pyogenes*) or pRT1-Clo051-dCas9 double NLS.

[00531] According to some embodiments, a Cas-CLOVER system comprises a fusion protein comprising a DNA localization component and an effector molecule, wherein the DNA localization component hybridizes to a target sequence of a DNA molecule in a TIL, wherein the DNA molecule encodes and the TIL expresses at least one immune checkpoint molecule, and the effector molecule cleaves the DNA molecule, whereby expression of the at least one immune checkpoint molecule is altered.

[00532] According to particular embodiments, a Cas-CLOVER method comprises silencing or reducing the expression of one or more immune checkpoint genes in TILs by introducing a Cas-CLOVER system (*e.g.*, dCas9-Clo051, dSaCas9-Clo051, Xanthomonas-TALE-Clo051, or Ralstonia-TALE-Clo051 fusion protein) specific to a target DNA sequence of the immune checkpoint gene(s). The fusion protein may be delivered as DNA, mRNA, protein. Upon contact of the genome with the Cas-CLOVER system, one or more strand of the target double-stranded DNA may be cut. If the cut is made in the presence of one or more DNA repair

pathways or components thereof, the Cas-CLOVER method either interrupts gene expression or modifies the genomic sequence by insertion, deletion, or substitution of one or more base pairs. DSBs may be repaired in the cells by non-homologous end joining (NHEJ), a mechanism which frequently causes insertions or deletions (indels) in the DNA. Indels often lead to frameshifts, creating loss of function alleles; for example, by causing premature stop codons within the open reading frame (ORF) of the targeted gene. According to certain embodiments, the result is a loss-of-function mutation within the targeted immune checkpoint gene.

[00533] Alternatively, DSBs induced by Cas-CLOVER systems may be repaired by homology-directed repair (HDR) instead of NHEJ. While NHEJ-mediated DSB repair often disrupts the open reading frame of the gene, homology directed repair (HDR) can be used to generate specific nucleotide changes ranging from a single nucleotide change to large insertions. According to some embodiments, HDR is used for gene editing immune checkpoint genes by delivering a DNA repair template containing the desired sequence into the TILs with the Cas-CLOVER system. The repair template preferably contains the desired edit as well as additional homologous sequence immediately upstream and downstream of the target gene (often referred to as left and right homology arms).

[00534] Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a Cas-CLOVER method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGF β , PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, TET2, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, TOX, SOCS1, ANKRD11, and BCOR.

[00535] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a Cas-CLOVER method, and which may be used in accordance with embodiments of the present invention, are described in WO2019126578, US2017/0107541, US2017/0114149, US2018/0187185, and U.S. Patent No. 10,415,024, the contents of which are incorporated herein by reference in their entirety. Resources for carrying out Cas-CLOVER

methods, such as CLOVER mRNA and Cas-CLOVER mRNA constructs, are commercially available from companies such as Demeetra and Hera Biolabs.

a. piggyBac Methods

[00536] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a piggyBac method (*e.g.*, piggyBac transposons and transposases or piggyBac-like transposons and transposases). According to particular embodiments, the use of a piggyBac method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface of at least a portion of the therapeutic population of TILs. Alternatively, the use of a piggyBac method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface of, and optionally causes one or more immune checkpoint genes to be enhanced in, at least a portion of the therapeutic population of TILs. In some embodiments, the at least one immunomodulatory composition comprises an immunomodulatory agent fused to a membrane anchor (*e.g.*, a membrane anchored immunomodulatory fusion protein described herein). In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-2, IL-7, IL-10, IL-12, IL-15, IL-18, IL-21, and a CD40 agonist (*e.g.*, a CD40L or an agonistic CD40 binding domain). In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-2, IL-12, IL-15, IL-18, IL-21, and a CD40 agonist. In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-12, IL-15, IL-18, IL-21, and a CD40 agonist.

[00537] The piggyBac transposon is a mobile genetic element that efficiently transposes between the donor vector and host chromosomes. This system has almost no cargo limit, and is fully reversible, leaving no footprint in the genome after excision. The piggyBac transposon/transposase system consists of a transposase that recognizes piggyBac-specific inverted terminal repeat sequences (ITRs) located on both sides of the transposon cassette. The transposase excises the transposable element to integrate it into TT/AA chromosomal sites that are preferentially located in euchromatic regions of mammalian genomes (Ding *et al.* 2005; Cadinaños and Bradley 2007; Wilson *et al.* 2007; Wang *et al.* 2008; Li *et al.* 2011).

[00538] Exemplary piggyBac systems include those described in WO2019/046815, the contents of which are incorporated herein by reference in their entirety. In embodiments, the piggyBac system comprises a transposon/transposase system.

[00539] In embodiments, a piggyBac method comprises delivering to the TILs, (a) a nucleic acid or amino acid sequence comprising a sequence encoding a transposase enzyme and (b) a recombinant and non-naturally occurring DNA sequence comprising a DNA sequence encoding a transposon.

[00540] In embodiments, the sequence encoding a transposase enzyme is an mRNA sequence. In embodiments, the sequence encoding a transposase enzyme is a DNA sequence. In embodiments, the DNA sequence is a cDNA sequence. In embodiments, the sequence encoding a transposase enzyme is an amino acid sequence. A protein Super piggybac transposase (SPB) may be delivered following pre-incubation with transposon DNA.

[00541] Transposons/Transposases

[00542] Exemplary transposon/transposase systems include, but are not limited to, piggyBac transposons and transposases, Sleeping Beauty transposons and transposases, Helraiser transposons and transposases and Tol2 transposons and transposases.

[00543] The piggyBac transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) on the ends of the transposon, and moves the contents between the ITRs into TTAA chromosomal sites. The piggyBac transposon system has no payload limit for the genes of interest that can be included between the ITRs. In embodiments, the transposon is a piggyBac transposon or a piggyBac-like transposon.

[00544] Examples of piggyBac and piggyBac-like transposases and transposons include, for example, those disclosed in WO2019/046815, the contents of which are incorporated herein by reference in their entirety. In embodiments, the piggyBac or piggyBac-like transposase is hyperactive. A hyperactive piggyBac or piggyBac-like transposase is a transposase that is more active than the naturally occurring variant from which it is derived. In embodiments, the hyperactive piggyBac or piggyBac-like transposase enzyme is isolated or derived from *Bombyx mori*. A list of hyperactive amino acid substitutions can be found in US patent No. 10,041,077, the contents of which are incorporated herein by reference in their entirety. In embodiments, the

piggyBac or piggyBac-like transposase is integration deficient. In embodiments, an integration deficient piggyBac or piggyBac-like transposase is a transposase that can excise its corresponding transposon, but that integrates the excised transposon at a lower frequency than a corresponding wild-type transposase. A list of integration deficient amino acid substitutions can be found in US patent No. 10,041,077, the contents of which are incorporated by reference in their entirety.

[00545] In embodiments, the piggyBac or piggyBac-like transposon is capable of insertion by a piggyBac or piggyBac-like transposase at the sequence 5'-TTAT-3' within a target nucleic acid. In embodiments, and, in particular, embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac transposase. In embodiments, and, in particular, embodiments wherein the transposon is a piggyBac-like transposon, the transposase is a piggyBac-like transposase. In embodiments, and, in particular, embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac™ or a Super piggyBac™ (SPB) transposase. In embodiments, and, in particular, embodiments wherein the transposase is a Super piggyBac™ (SPB) transposase, the sequence encoding the transposase is an mRNA sequence.

[00546] The sleeping beauty (SB) transposon is transposed into the target genome by the Sleeping Beauty transposase that recognizes ITRs, and moves the contents between the ITRs into TA chromosomal sites. In embodiments, the transposon is a Sleeping Beauty transposon. In embodiments, the transposase enzyme is a Sleeping Beauty transposase enzyme (see, for example, US Patent No. 9,228,180, the contents of which are incorporated herein in their entirety). In embodiments, the Sleeping Beauty transposase is a hyperactive Sleeping Beauty (SB100X) transposase.

[00547] The Helraiser transposon is transposed by the Helitron transposase. Unlike other transposases, the Helitron transposase does not contain an RNase-H like catalytic domain, but instead comprises a RepHel motif made up of a replication initiator domain (Rep) and a DNA helicase domain. The Rep domain is a nuclease domain of the HUH superfamily of nucleases. In embodiments, the transposon is a Helraiser transposon. In embodiments of the Helraiser transposon sequence, the transposase is flanked by left and right terminal sequences termed LTS and RTS. In embodiments, these sequences terminate with a conserved 5'-TC/CTAG-3' motif.

In embodiments, a 19 bp palindromic sequence with the potential to form the hairpin termination structure is located 11 nucleotides upstream of the RTS and comprises the sequence GTGCACGAATTTTCGTGCACCGGGCCACTAG. In embodiments, and, in particular embodiments wherein the transposon is a Helraiser transposon, the transposase enzyme is a Helitron transposase enzyme.

[00548] Tol2 transposons may be isolated or derived from the genome of the medaka fish, and may be similar to transposons of the hAT family. Exemplary Tol2 transposons of the disclosure are encoded by a sequence comprising about 4.7 kilobases and contain a gene encoding the Tol2 transposase, which contains four exons. In embodiments, the transposon is a Tol2 transposon. In certain embodiments of the methods of the disclosure, and, in particular those embodiments wherein the transposon is a Tol2 transposon, the transposase enzyme is a Tol2 transposase enzyme.

[00549] In embodiments, a vector comprises the recombinant and non-naturally occurring DNA sequence encoding the transposon. In embodiments, the vector comprises any form of DNA and wherein the vector comprises at least 100 nucleotides (nts), 500 nts, 1000 nts, 1500 nts, 2000 nts, 2500 nts, 3000 nts, 3500 nts, 4000 nts, 4500 nts, 5000 nts, 6500 nts, 7000 nts, 7500 nts, 8000 nts, 8500 nts, 9000 nts, 9500 nts, 10,000 nts or any number of nucleotides in between. In embodiments, the vector comprises single-stranded or double-stranded DNA. In embodiments, the vector comprises circular DNA. In embodiments, the vector is a plasmid vector, a nanoplasmid vector, a minicircle. In embodiments, the vector comprises linear or linearized DNA. In embodiments, the vector is a double-stranded doggybone™ DNA sequence.

[00550] In embodiments, the recombinant and non-naturally occurring DNA sequence encoding a transposon further comprises a sequence encoding one or more immune checkpoint genes.

[00551] In embodiments, the nucleic acid sequence encoding the transposase enzyme is a DNA sequence, and an amount of the DNA sequence encoding the transposase enzyme and an amount of the DNA sequence encoding the transposon is equal to or less than 10.0 µg per 100 µL, less than 7.5 µg per 100 µL, less than 6.0 µg per 100 µL, less than 5.0 µg per 100 µL, less than 2.5 µg per 100 µL, or less than 1.67 µg per 100 µL, less than 0.55 µg per 100 µL, less than 0.19 µg per 100 µL, less than 0.10 µg per 100 µL of an electroporation or nucleofection reaction.

In certain embodiments, a concentration of the amount of the DNA sequence encoding the transposase enzyme and an amount of the DNA sequence encoding the transposon in the electroporation or nucleofection reaction is equal to or less than 100 µg/mL, equal to or less than 75 µg/mL, equal to or less than 60 µg/mL, equal to or less than 50 µg/mL, equal to or less than 25 µg/mL, equal to or less than 16.7 µg/mL, equal to or less than 5.5 µg/mL, equal to or less than 1.9 µg/mL, equal to or less than 1.0 µg/mL.

[00552] In embodiments, the nucleic acid sequence encoding the transposase enzyme is an RNA sequence, and an amount of the RNA sequence encoding the transposase enzyme and an amount of the RNA sequence encoding the transposon is equal to or less than 10.0 µg per 100 µL, less than 7.5 µg per 100 µL, less than 6.0 µg per 100 µL, less than 5.0 µg per 100 µL, less than 2.5 µg per 100 µL, or less than 1.67 µg per 100 µL, less than 0.55 µg per 100 µL, less than 0.19 µg per 100 µL, less than 0.10 µg per 100 µL of an electroporation or nucleofection reaction. In certain embodiments, a concentration of the amount of the RNA sequence encoding the transposase enzyme and an amount of the RNA sequence encoding the transposon in the electroporation or nucleofection reaction is equal to or less than 100 µg/mL, equal to or less than 75 µg/mL, equal to or less than 60 µg/mL, equal to or less than 50 µg/mL, equal to or less than 25 µg/mL, equal to or less than 16.7 µg/mL, equal to or less than 5.5 µg/mL, equal to or less than 1.9 µg/mL, equal to or less than 1.0 µg/mL.

[00553] In embodiments, the TILs are further modified by a second gene editing tool, including, but not limited to those described herein. In embodiments, the second gene editing tool may include an excision-only piggyBac transposase to re-excise the inserted sequences or any portion thereof. For example, the excision-only piggyBac transposase may be used to "re-excise" the transposon.

[00554] According to some embodiments, a piggyBac system comprises a transposon/transposase system, wherein the transposase recognizes the ITRs located on both sides of the transposon cassette comprising a cargo encoding one or more immune checkpoint genes, and excises the transposable element to integrate it into TT/AA chromosomal sites, resulting in genomic insertion of the transposon cassette and expression of the one or more immune checkpoint genes. According to some embodiments, the cargo encodes two or more immune checkpoint molecules.

[00555] Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a piggyBac method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-18, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00556] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a piggyBac method, and which may be used in accordance with embodiments of the present invention, are described in WO2019/046815, WO2015006700, WO2010085699, WO2010099301, WO2010099296, WO2006122442, WO2001081565, and WO1998040510, the contents of which are incorporated herein by reference in their entirety.

[00557] Resources for carrying out piggyBac methods, such as plasmids for expressing transposons/transposases, are commercially available from companies such as Demeetra and Hera Biolabs.

[00558] In some embodiments, a method of genetically modifying a population of TILs includes the use of a non-viral technique such as a piggyBac method (*e.g.*, piggyBac transposons and transposases or piggyBac-like transposons and transposases). In some embodiments, the method comprises delivering to the TILs: (a) a nucleic acid or amino acid sequence comprising a sequence encoding a transposase enzyme; and (b) a recombinant and non-naturally occurring DNA sequence comprising a DNA sequence encoding a transposon. In certain embodiments of the methods of the disclosure, the sequence encoding a transposase enzyme is an mRNA sequence. The mRNA sequence encoding a transposase enzyme may be produced in vitro. In certain embodiments of the methods of the disclosure, the sequence encoding a transposase enzyme is a DNA sequence. The DNA sequence encoding a transposase enzyme may be produced in vitro. The DNA sequence may be a cDNA sequence. In certain embodiments of the methods of the disclosure, the sequence encoding a transposase enzyme is an amino acid sequence. The amino acid sequence encoding a transposase enzyme may be produced in vitro. A protein Super piggybac transposase (SPB) may be delivered following pre-incubation with transposon DNA. In certain embodiments, the transposon is a piggyBac transposon or a piggyBac-like transposon. In certain embodiments, and, in particular, those embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac transposase. In certain embodiments, and, in particular, those embodiments wherein the transposon is a piggyBac-like transposon, the transposase is a piggyBac-like transposase. In certain

embodiments, the piggyBac transposase comprises an amino acid sequence comprising SEQ ID NO: 14487 of WO2019046815. In certain embodiments, and, in particular, those embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac™ or a Super piggyBac™ (SPB) transposase. In certain embodiments, and, in particular, those embodiments wherein the transposase is a Super piggyBac™ (SPB) transposase, the sequence encoding the transposase is an mRNA sequence. In certain embodiments of the methods of the disclosure, the transposase enzyme is a piggyBac™ (PB) transposase enzyme. The piggyBac (PB) transposase enzyme may comprise or consist of an amino acid sequence at least 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between identical to:

1 MGSSLDDEHI LSALLQSDDE LVGEDSDSEI SDHVSEDDVQ SDTEEFIDE
VHEVQPTSSG

61 SEILDEQNV I EQPGSSLASN RILTLPQRTI RGKNKHCWST SKSTRRSRVS
ALNIVRSQRG

121 PTRMCRNIYD PLLCFKLFFT DEIISEIVKW TNAEISLKRR ESMTGATFRD
TNEDEIYAFF

181 GILVMTAVRK DNHMSTDDL FDRSLSMVYVS VMSRDRFDFL IRCLRMDDKS
IRPTLRENDV

241 FTPVRKIWDL FIHQCIQNYT PGAHLTIDEQ LLGFRGRCPF RMYIPNKPSK
YGIKILMMCD

301 SGTKYMINGM PYLGRGTQTN GVPLGEYYVK ELSKPVHGSC RNITCDNWFT
SIPLAKNLLQ

361 EPYKLTIVGT VRSNKREIPE VLKNSRSRPV GTSMFCFDGP LTLVSYKPKP
AKMVYLLSSC

421 DEDASINEST GKPQMVMYYN QTKGGVDTLD QMCSVMTCR KTNRWPMALL
YGMINIACIN

481 SFIIYSHNV SSKGEKVQSRK KFMRNLYMSL TSSFMRKRLE APTLKRYLRD
NISNILPNEV

541 PGTSDSTEE PVMKKRTYCT YCPSKIRRKA NASCKKCKKV ICREHNIDMC QSCF
(SEQ ID NO:239; SEQ ID NO: 14487 of WO2019046815).

[00559] In certain embodiments of the methods of the disclosure, the transposon is a Sleeping Beauty transposon. In certain embodiments of the methods of the disclosure, the transposase enzyme is a Sleeping Beauty transposase enzyme (see, for example, US Patent No. 9,228,180, the contents of which are incorporated herein in their entirety). In certain embodiments, the Sleeping Beauty transposase is a hyperactive Sleeping Beauty (SB100X) transposase. In certain embodiments, the Sleeping Beauty transposase enzyme comprises an amino acid sequence at least 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between identical to:

1 MGKSKEISQD LRKKIVDLHK SGSSLGAISK RLKVPRSSVQ TIVRKYKHHG
TTQPSYRSGR

61 RRVLSRDER TLVRKVQINP RTTAKDLVKM LEETGTKVSI STVKRVLYRH
NLKGRSARKK

121 PLLQNRHKKA RLRFATAHGD KDRTFWRNVL WSDETKIELF GHNDHRYVWR
KKGEACKPKN

181 TIPTVKHGGG SIMLWGCFAA GGTGALHKID GIMRKENYVD ILKQHLKTSV
RKLLKGRK V

241 FQMDNDPKHT SKWAKWLKD NKVKVLEWPS QSPDLNPIEN LWAEKKRVR
ARRPTNLTQL

301 HQLCQEEWAK IHPTYCGKLV EGYPKRLTQV KQFKGNATKY (SEQ ID NO:240;
SEQ ID NO: 14485 of WO2019046815).

[00560] In certain embodiments, including those wherein the Sleeping Beauty transposase is a hyperactive Sleeping Beauty (SB100X) transposase, the Sleeping Beauty transposase enzyme comprises an amino acid sequence at least at least 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between identical to:

1 MGKSKEISQD LRKRIVDLHK SGSSLGAISK RLAVPRSSVQ TIVRKYKHHG
TTQPSYRSGR

61 RRVLSRDER TLVRKVQINP RTTAKDLVKM LEETGTKVSI STVKRVL YRH
NLKGHSARKK

121 PLLQNRHKKA RLRFATAHGD KDRTFWRNVL WSEDTKIELF GHNDHRYVWR
KKGEACKPKN

181 TIPTVKHGGG SIMLWGCFAA GGTGALHKID GIMDAVQYVD ILKQHLKTSV
RKLKLGRKWV

241 FQHDNDPKHT SKWAKWLKD NKVKVLEWPS QSPDLNPIEN LWAEKKRVR
ARRPTNLTQL

301 HQLCQEEWAK IHPNYCGKLV EGYPKRLTQV KQFKGNATKY (SEQ ID NO:241;
SEQ ID NO: 14486 of WO2019046815).

[00561]

F. Rapid Second Expansion

[00562] The enriched tumor reactive TILs may be further expanded. In some embodiments, the population of TILs comprising the plurality of tumor reactive TILs may be further expanded. In some embodiments, the plurality of tumor reactive TILs is further expanded after being separated from the non-tumor reactive TILs.

[00563] This further expansion is referred to herein as the rapid second expansion or a rapid expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (Rapid Expansion Protocol or REP). The rapid second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container. In some embodiments, 1 day, 2 days, 3 days, or 4 days after initiation of the rapid second expansion, the TILs are transferred to a larger volume container.

[00564] In some embodiments, the rapid second expansion (which can include expansions sometimes referred to as REP) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days or longer after initiation of the rapid second expansion. In some

[illegible]

the second TIL expansion can proceed for about 5 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 11 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 12 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 13 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 14 days after initiation of the rapid second expansion.

[00565] In some embodiments, the rapid second expansion can be performed in a gas permeable container using the methods of the present disclosure (including, for example, expansions referred to as REP). In some embodiments, the TILs are expanded in the rapid second expansion in the presence of IL-2, OKT-3, and feeder cells (also referred herein as “antigen-presenting cells”). In some embodiments, the TILs are expanded in the rapid second expansion in the presence of IL-2, OKT-3, and feeder cells, wherein the feeder cells are added to a final concentration that is twice, 2.4 times, 2.5 times, 3 times, 3.5 times or 4 times the concentration of feeder cells present in the first expansion. For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/mL of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San Diego, CA, USA). TILs can be expanded to induce further stimulation of the TILs *in vitro* by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 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.

[00566] In some embodiments, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In some embodiments, 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 some embodiments, 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.

[00567] In some embodiments, 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 some embodiments, 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 some embodiments, 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 comprises between 15 ng/mL and 30 ng/mL of OKT-3 antibody. In some

embodiments, the cell culture medium comprises between 30 ng/mL and 60 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL OKT-3. In some embodiments, the cell culture medium comprises about 60 ng/mL OKT-3. In some embodiments, the OKT-3 antibody is muromonab.

[00568] In some embodiments, the media in the rapid second expansion comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media in the rapid second expansion comprises antigen-presenting feeder cells. In some embodiments, the media in the rapid second expansion comprises 7.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media in the rapid second expansion comprises OKT-3. In some embodiments, the in the rapid second expansion media comprises 500 mL of culture medium and 30 μ g of OKT-3 per container. In some embodiments, the container is a G-REX-100 MCS flask. In some embodiments, the in the rapid second expansion media comprises 6000 IU/mL of IL-2, 60 ng/mL of OKT-3, and 7.5×10^8 antigen-presenting feeder cells. In some embodiments, the media comprises 500 mL of culture medium and 6000 IU/mL of IL-2, 30 μ g of OKT-3, and 7.5×10^8 antigen-presenting feeder cells per container.

[00569] In some embodiments, the media in the rapid second expansion comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media in the rapid second expansion comprises antigen-presenting feeder cells. In some embodiments, the media comprises between 5×10^8 and 7.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media in the rapid second expansion comprises OKT-3. In some embodiments, the media in the rapid second expansion comprises 500 mL of culture medium and 30 μ g of OKT-3 per container. In some embodiments, the container is a G-REX-100 MCS flask. In some embodiments, the media in the rapid second expansion comprises 6000 IU/mL of IL-2, 60 ng/mL of OKT-3, and between 5×10^8 and 7.5×10^8 antigen-presenting feeder cells. In some embodiments, the media in the rapid second expansion comprises 500 mL of culture medium and 6000 IU/mL of IL-2, 30 μ g of OKT-3, and between 5×10^8 and 7.5×10^8 antigen-presenting feeder cells per container.

[00570] 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.

[00571] 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.

[00572] 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 G processes according to Figure 5 (in particular, *e.g.*, Figure 5A and/or Figure 5B and/or Figure 5C), 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.

[00573] 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).

[00574] 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 some embodiments, the cell culture medium further comprises IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15.

[00575] 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 some embodiments, the cell culture medium further comprises IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21.

[00576] In some embodiments, the antigen-presenting feeder cells (APCs) are PBMCs. In some embodiments, the ratio of TILs to PBMCs and/or antigen-presenting cells in the rapid expansion and/or the second expansion is about 1 to 10, about 1 to 15, about 1 to 20, about 1 to 25, about 1 to 30, about 1 to 35, about 1 to 40, about 1 to 45, about 1 to 50, about 1 to 75, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In some embodiments, 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 some embodiments, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 100 and 1 to 200.

[00577] In some embodiments, REP and/or the rapid second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, wherein the feeder cell concentration is at least 1.1 times (1.1X), 1.2X, 1.3X, 1.4X, 1.5X, 1.6X, 1.7X, 1.8X, 1.8X, 2X, 2.1X, 2.2X, 2.3X, 2.4X, 2.5X, 2.6X, 2.7X, 2.8X, 2.9X, 3.0X, 3.1X, 3.2X, 3.3X, 3.4X, 3.5X, 3.6X, 3.7X, 3.8X, 3.9X or 4.0X the feeder cell concentration in the first expansion, 30 ng/mL OKT3 anti-CD3 antibody and 6000 IU/mL IL-2 in 150 mL media. Media replacement is done (generally 2/3 media replacement via aspiration of 2/3 of spent media and replacement with an equal volume of fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

[00578] In some embodiments, the rapid second expansion (which can include processes referred to as the REP process) is 7 to 9 days, as discussed in the examples and figures. In some embodiments, the second expansion is 7 days. In some embodiments, the second expansion is 8 days. In some embodiments, the second expansion is 9 days.

[00579] In some embodiments, the second expansion (which can include expansions referred to as REP) 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, 6000 IU per mL of IL-2, and added back to the original GREX-100 flasks. When TIL are expanded serially in GREX-100 flasks, on day 10 or 11 the TILs can be moved to a larger flask, such as a GREX-500. The cells may be harvested on day 14 of culture. The cells may be harvested on day 15 of culture. The cells may be harvested on day 16 of culture. In some embodiments, media replacement is done until the cells are transferred to an alternative growth chamber. In some embodiments, 2/3 of the media is replaced

by aspiration of spent media and replacement with an equal volume of fresh media. In some embodiments, alternative growth chambers include GREX flasks and gas permeable containers as more fully discussed below.

[00580] 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.

[00581] In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or serum replacement. In some embodiments, the basal cell medium includes, but is not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-Cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00582] 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⁺, Al³⁺, Ba²⁺, Cd²⁺, Co²⁺, Cr³⁺, Ge⁴⁺, Se⁴⁺, Br, T, Mn²⁺, P, Si⁴⁺, V⁵⁺, Mo⁶⁺, Ni²⁺, Rb⁺, Sn²⁺ and Zr⁴⁺. In some embodiments, the defined medium further comprises L- glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[00583] In some embodiments, the CTSTTMOpTmizerTM T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTSTTM OpTmizerTM T-cell Expansion Basal Medium, CTSTTM OpTmizerTM T-cell Expansion SFM, CTSTTM AIM-V Medium, CSTTM AIM-V SFM, LymphoONETM 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.

[00584] 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.

[00585] In some embodiments, the serum-free or defined medium is CTSTTM OpTmizerTM T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTSTTM OpTmizerTM is useful in the present invention. CTSTTM OpTmizerTM T-cell Expansion SFM is a combination of 1 L CTSTTM OpTmizerTM T-cell Expansion Basal Medium and 26 mL CTSTTM OpTmizerTM T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTSTTM OpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM.

[00586] In some embodiments, the defined medium is CTSTTM OpTmizerTM T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTSTTM OpTmizerTM is useful in the present invention. CTSTTM OpTmizerTM T-cell Expansion SFM is a combination of 1 L CTSTTM OpTmizerTM T-cell Expansion Basal Medium and 26 mL CTSTTM OpTmizerTM T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTSTTM OpTmizerTM T-cell Expansion SFM is supplemented with about 3% of the CTSTTM Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In

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

[00587] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (*i.e.*, GlutaMAX®) at a concentration of from about 0.1 mM to about 10 mM, 0.5mM to about 9 mM, 1 mM to about 8 mM, 2 mM to about 7 mM, 3 mM to about 6 mM, or 4 mM 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 2 mM.

[00588] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5 mM to about 150 mM, 10 mM to about 140 mM, 15 mM to about 130 mM, 20 mM to about 120 mM, 25 mM to about 110 mM, 30 mM to about 100 mM, 35 mM to about 95 mM, 40 mM to about 90 mM, 45 mM to about 85 mM, 50 mM to about 80 mM, 55 mM to about 75 mM, 60 mM to about 70 mM, or about 65 mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM.

[00589] In some embodiments, the defined media described in International Patent Application Publication No. WO1998/030679 and U.S. Patent Application Publication No. US 2002/0076747 A1, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum-free culture. The serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L- isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine,

L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^+ , Al^{3+} , Ba^{2+} , Cd^{2+} , Co^{2+} , Cr^{3+} , Ge^{4+} , Se^{4+} , Br, T, Mn^{2+} , P, Si^{4+} , V^{5+} , Mo^{6+} , Ni^{2+} , Rb^+ , Sn^{2+} and Zr^{4+} . In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (α MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00590] 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.

[00591] 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 5. 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 5. 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 5.

[00592] In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μ M), 2-mercaptoethanol (final concentration of about 100 μ M).

[00593] In some embodiments, the defined media described in Smith, *et al.*, *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.

[00594] In some embodiments, 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 some embodiments, 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).

[00595] In some embodiments, the rapid second expansion (including expansions referred to as REP) is performed and further comprises a step wherein TILs are selected for superior tumor reactivity. Any selection method known in the art may be used. For example, the methods described in U.S. Patent Application Publication No. 2016/0010058 A1, the disclosures of which are incorporated herein by reference, may be used for selection of TILs for superior tumor reactivity.

[00596] Optionally, a cell viability assay can be performed after the rapid second expansion (including expansions referred to as the REP expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. In some embodiments, TIL samples can be counted and viability determined using a Cellometer K2 automated cell counter (Nexcelom Bioscience, Lawrence, MA). In some embodiments, viability is determined according to the standard Cellometer K2 Image Cytometer Automatic Cell Counter protocol.

[00597] 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 heavy chain. In some embodiments, the diversity 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).

[00598] In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below. In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises 6000 IU/mL IL-2, 30 ug/flask OKT-3, as well as 7.5×10^8 antigen-presenting feeder cells (APCs), as discussed in more detail below. In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below. In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises 6000 IU/mL IL-2, 30 ug/flask OKT-3, as well as 5×10^8 antigen-presenting feeder cells (APCs), as discussed in more detail below.

[00599] In some embodiments, the rapid second expansion is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-100 or a G-REX-500. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-500.

[00600] In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (a) performing the rapid second expansion by culturing TILs in a small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period of about 3 to 7 days, and then (b) effecting the transfer of the TILs in the small scale culture to a second container larger than the first container, *e.g.*, a G-REX-500-MCS container, and culturing the TILs from the small scale culture in a larger scale culture in the second container for a period of about 4 to 7 days.

[00601] In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling out of the culture by: (a) performing the rapid second expansion by culturing TILs in a first small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period of about 3 to 7 days, and then (b) effecting the transfer and apportioning of the TILs from the first small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are equal in size to the first container, wherein in each second container the portion of the TILs from first small scale culture transferred to such second container is cultured in a second small scale culture for a period of about 4 to 7 days.

[00602] In some embodiments, the first small scale TIL culture is apportioned into a plurality of about 2 to 5 subpopulations of TILs.

[00603] In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing TILs in a small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period of about 3 to 7 days, and then (b) effecting the transfer and apportioning of the TILs from the small scale culture into and amongst at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 second containers that are larger in size than the first container,

e.g., G-REX-500MCS containers, wherein in each second container the portion of the TILs from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 4 to 7 days.

[00604] In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid or second expansion by culturing TILs in a small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period of about 5 days, and then (b) effecting the transfer and apportioning of the TILs from the small scale culture into and amongst 2, 3 or 4 second containers that are larger in size than the first container, *e.g.*, G-REX-500 MCS containers, wherein in each second container the portion of the TILs from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 6 days.

[00605] In some embodiments, upon the splitting of the rapid second expansion, each second container comprises at least 10^8 TILs. In some embodiments, upon the splitting of the rapid or second expansion, each second container comprises at least 10^8 TILs, at least 10^9 TILs, or at least 10^{10} TILs. In one exemplary embodiment, each second container comprises at least 10^{10} TILs.

[00606] In some embodiments, the first small scale TIL culture is apportioned into a plurality of subpopulations. In some embodiments, the first small scale TIL culture is apportioned into a plurality of about 2 to 5 subpopulations. In some embodiments, the first small scale TIL culture is apportioned into a plurality of about 2, 3, 4, or 5 subpopulations.

[00607] In some embodiments, after the completion of the rapid second expansion, the plurality of subpopulations comprises a therapeutically effective amount of TILs. In some embodiments, after the completion of the rapid or second expansion, one or more subpopulations of TILs are pooled together to produce a therapeutically effective amount of TILs. In some embodiments, after the completion of the rapid expansion, each subpopulation of TILs comprises a therapeutically effective amount of TILs.

[00608] In some embodiments, the rapid second expansion is performed for a period of about 3 to 7 days before being split into a plurality of steps. In some embodiments, the splitting of the rapid second expansion occurs at about day 3, day 4, day 5, day 6, or day 7 after the initiation of the rapid or second expansion.

[00609] In some embodiments, the splitting of the rapid second expansion occurs at about day 7, day 8, day 9, day 10, day 11, day 12, day 13, day 14, day 15, or day 16 day 17, or day 18 after the initiation of the first expansion (*i.e.*, pre-REP expansion). In one exemplary embodiment, the splitting of the rapid or second expansion occurs at about day 16 after the initiation of the first expansion.

[00610] In some embodiments, the rapid second expansion is further performed for a period of about 7 to 11 days after the splitting. In some embodiments, the rapid second expansion is further performed for a period of about 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, or 11 days after the splitting.

[00611] In some embodiments, the cell culture medium used for the rapid second expansion before the splitting comprises the same components as the cell culture medium used for the rapid second expansion after the splitting. In some embodiments, the cell culture medium used for the rapid second expansion before the splitting comprises different components from the cell culture medium used for the rapid second expansion after the splitting.

[00612] In some embodiments, the cell culture medium used for the rapid second expansion before the splitting comprises IL-2, optionally OKT-3 and further optionally APCs. In some embodiments, the cell culture medium used for the rapid second expansion before the splitting comprises IL-2, OKT-3, and further optionally APCs. In some embodiments, the cell culture medium used for the rapid second expansion before the splitting comprises IL-2, OKT-3 and APCs.

[00613] In some embodiments, the cell culture medium used for the rapid second expansion before the splitting is generated by supplementing the cell culture medium in the first expansion with fresh culture medium comprising IL-2, optionally OKT-3 and further optionally APCs. In some embodiments, the cell culture medium used for the rapid second expansion before the splitting is generated by supplementing the cell culture medium in the first expansion with fresh culture medium comprising IL-2, OKT-3 and APCs. In some embodiments, the cell culture medium used for the rapid second expansion before the splitting is generated by replacing the cell culture medium in the first expansion with fresh cell culture medium comprising IL-2, optionally OKT-3 and further optionally APCs. In some embodiments, the cell culture medium used for the rapid second expansion before the splitting is generated by replacing the cell culture

medium in the first expansion with fresh cell culture medium comprising IL-2, OKT-3 and APCs.

[00614] In some embodiments, the cell culture medium used for the rapid second expansion after the splitting comprises IL-2, and optionally OKT-3. In some embodiments, the cell culture medium used for the rapid second expansion after the splitting comprises IL-2, and OKT-3. In some embodiments, the cell culture medium used for the rapid second expansion after the splitting is generated by replacing the cell culture medium used for the rapid second expansion before the splitting with fresh culture medium comprising IL-2 and optionally OKT-3. In some embodiments, the cell culture medium used for the rapid second expansion after the splitting is generated by replacing the cell culture medium used for the rapid second expansion before the splitting with fresh culture medium comprising IL-2 and OKT-3.

1. Feeder Cells and Antigen Presenting Cells

[00615] In some embodiments, the rapid second expansion procedures described herein (for example including those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the rapid second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

[00616] In general, the allogeneic 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.

[00617] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells on day 7 or 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

[00618] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second

expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody and 3000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 60 ng/mL OKT3 antibody and 6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 60 ng/mL OKT3 antibody and 3000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody and 6000 IU/mL IL-2.

[00619] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/mL OKT3 antibody and 1000-6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/mL OKT3 antibody and 2000-5000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/mL OKT3 antibody and 2000-4000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/mL OKT3 antibody and 2500-3500 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/mL OKT3 antibody and 6000 IU/mL IL-2.

[00620] 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 some embodiments, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 10, about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In some embodiments, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In some embodiments, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

[00621] In some embodiments, the second expansion procedures described herein require a ratio of about 5×10^8 feeder cells to about 100×10^6 TILs. In some embodiments, the second expansion procedures described herein require a ratio of about 7.5×10^8 feeder cells to about 100

$\times 10^6$ TILs. In other embodiments, the second expansion procedures described herein require a ratio of about 5×10^8 feeder cells to about 50×10^6 TILs. In other embodiments, the second expansion procedures described herein require a ratio of about 7.5×10^8 feeder cells to about 50×10^6 TILs. In yet other embodiments, the second expansion procedures described herein require about 5×10^8 feeder cells to about 25×10^6 TILs. In yet other embodiments, the second expansion procedures described herein require about 7.5×10^8 feeder cells to about 25×10^6 TILs. In yet other embodiments, the rapid second expansion requires twice the number of feeder cells as the rapid second expansion. In yet other embodiments, when the first expansion described herein requires about 2.5×10^8 feeder cells, the rapid second expansion requires about 5×10^8 feeder cells. In yet other embodiments, when the first expansion described herein requires about 2.5×10^8 feeder cells, the rapid second expansion requires about 7.5×10^8 feeder cells. In yet other embodiments, the rapid second expansion requires two times (2.0X), 2.5X, 3.0X, 3.5X or 4.0X the number of feeder cells as the first expansion.

[00622] In some embodiments, the rapid second expansion procedures described herein require an excess of feeder cells during the rapid second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, artificial antigen-presenting (aAPC) cells are used in place of PBMCs. In some embodiments, the PBMCs are added to the rapid second expansion at twice the concentration of PBMCs that were added to the first expansion.

[00623] In general, the allogeneic 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.

[00624] In some embodiments, artificial antigen presenting cells are used in the rapid second expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines and Other Additives

[00625] The rapid second expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

[00626] Alternatively, using combinations of cytokines for the rapid second expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is described in U.S. Patent Application Publication No. US 2017/0107490 A1, the disclosure of which is incorporated by reference herein. 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.

[0004] In some embodiments, the second expansion may also include the addition of OKT-3 antibody or muromonab to the culture media, as described elsewhere herein. In some embodiments, the second expansion may also include the addition of a 4-1BB agonist to the culture media, as described elsewhere herein. In some embodiments, the second expansion may also include the addition of an OX-40 agonist to the culture media, as described elsewhere herein. In addition, additives such as peroxisome proliferator-activated receptor gamma coactivator I-alpha agonists, including proliferator-activated receptor (PPAR)-gamma agonists such as a thiazolidinedione compound, may be used in the culture media during the second expansion, as described in U.S. Patent Application Publication No. US 2019/0307796 A1, the disclosure of which is incorporated by reference herein.

G. Harvesting TILs

[00627] After the rapid second expansion step, cells can be harvested.

[00628] TILs can be harvested in any appropriate and sterile manner, including, for example by centrifugation. Methods for TIL harvesting are well known in the art and any such known methods can be employed with the present process. In some embodiments, TILs are harvested using an automated system.

[00629] Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell-based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing system is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term “LOVO cell processing system” also refers to any instrument or device manufactured by any vendor that can

pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

[00630] In some embodiments, TILs are harvested according to the methods described in herein. In some embodiments, TILs between days 14 and 16 are harvested using the methods as described herein. In some embodiments, TILs are harvested at 14 days using the methods as described herein. In some embodiments, TILs are harvested at 15 days using the methods as described herein. In some embodiments, TILs are harvested at 16 days using the methods as described herein.

H. Final Formulation and Transfer to Infusion Container

[00631] After harvesting, TILs are transferred to a container for use in administration to a patient, such as an infusion bag or sterile vial. 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.

[00632] In some embodiments, TILs expanded using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In some embodiments, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded as disclosed herein may be administered by any suitable route as known in the art. In some embodiments, the TILs are administered as a single intra-arterial or intravenous infusion, which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic administration.

[00633] The expanded population of TILs (for example after the second expansion) 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, 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.

[00634] 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.

[00635] In some embodiments, a population of TILs is cryopreserved using CS10 cryopreservation media (CryoStor 10, BioLife Solutions). In some embodiments, a population of TILs is cryopreserved using a cryopreservation media containing dimethylsulfoxide (DMSO). In some embodiments, a population of TILs is cryopreserved using a 1:1 (vol:vol) ratio of CS10 and cell culture media. In some embodiments, 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.

[00636] As discussed above, cryopreservation can occur at numerous points throughout the TIL expansion process. In some embodiments, the expanded population of TILs after the first expansion can be cryopreserved. Cryopreservation can be generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See Sadeghi, *et al.*, *Acta Oncologica* 2013, 52, 978-986. In some embodiments, the TILs are cryopreserved in 5% DMSO. In some embodiments, the TILs are cryopreserved in cell culture media plus 5% DMSO. In some embodiments, the TILs are cryopreserved according to the methods provided in Example 5.

[00637] 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.

III. Pharmaceutical Compositions, Dosages, and Dosing Regimens

[00638] In some embodiments, TILs, MILs, or PBLs expanded and/or genetically modified (including TILs, MILs, or PBLs genetically modified to express a CCR) using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In some embodiments, 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.

[00639] 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 NSCLC or melanoma. In some embodiments, about 1.2×10^{10} to about 4.3×10^{10} of TILs are administered. In some embodiments, about 3×10^{10} to about 12×10^{10} TILs are administered. In some embodiments, about 4×10^{10} to about 10×10^{10} TILs are administered. In some embodiments, about 5×10^{10} to about 8×10^{10} TILs are administered. In some embodiments, about 6×10^{10} to about 8×10^{10} TILs are administered. In some embodiments, about 7×10^{10} to about 8×10^{10} TILs are administered. In some embodiments, the therapeutically effective dosage is about 2.3×10^{10} to about 13.7×10^{10} . In some embodiments, the therapeutically effective dosage is about 7.8×10^{10} TILs, particularly of the cancer is melanoma. In some embodiments, the therapeutically effective dosage is about 7.8×10^{10} TILs, particularly of the cancer is NSCLC. 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. In some embodiments, the therapeutically effective dosage is about 1×10^9 to about 1×10^{11} TILs.

[00640] 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 some embodiments, 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} .

[00641] 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.

[00642] 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.

[00643] 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.

[00644] 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.

[00645] 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.

[00646] 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.

[00647] 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.

[00648] 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.

[00649] 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} .

[00650] 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.

[00651] 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.

[00652] 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.

[00653] In other embodiments, the invention provides an infusion bag comprising the therapeutic population of TILs described in any of the preceding paragraphs above.

[00654] In other embodiments, the invention provides a tumor infiltrating lymphocyte (TIL) composition comprising the therapeutic population of TILs described in any of the preceding paragraphs above and a pharmaceutically acceptable carrier.

[00655] In other embodiments, the invention provides an infusion bag comprising the TIL composition described in any of the preceding paragraphs above.

[00656] In other embodiments, the invention provides a cryopreserved preparation of the therapeutic population of TILs described in any of the preceding paragraphs above.

[00657] In other embodiments, the invention provides a tumor infiltrating lymphocyte (TIL) composition comprising the therapeutic population of TILs described in any of the preceding paragraphs above and a cryopreservation media.

[00658] In other embodiments, the invention provides the TIL composition described in any of the preceding paragraphs above modified such that the cryopreservation media contains DMSO.

[00659] In other embodiments, the invention provides the TIL composition described in any of the preceding paragraphs above modified such that the cryopreservation media contains 7-10% DMSO.

[00660] In other embodiments, the invention provides a cryopreserved preparation of the TIL composition described in any of the preceding paragraphs above.

[00661] In some embodiments, TILs expanded using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In some embodiments, 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.

[00662] 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 NSCLC. In some embodiments, 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, therapeutically effective dosage is about 2.3×10^{10} to about 13.7×10^{10} . In some embodiments, therapeutically effective dosage is about 7.8×10^{10} TILs, particularly of the cancer is NSCLC. In some embodiments, therapeutically effective dosage is about 1.2×10^{10} to about 4.3×10^{10} of TILs. In some embodiments, therapeutically effective dosage is about 3×10^{10} to about 12×10^{10} TILs. In some embodiments, therapeutically effective dosage is about 4×10^{10} to about 10×10^{10} TILs. In some embodiments, therapeutically effective dosage is about 5×10^{10} to about 8×10^{10} TILs. In some embodiments, therapeutically effective dosage is about 6×10^{10} to about 8×10^{10} TILs. In some embodiments, therapeutically effective dosage is about 7×10^{10} to about 8×10^{10} TILs.

[00663] 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 some embodiments, 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} .

[00664] 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.

[00665] 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.

[00666] 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.

[00667] 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.

[00668] 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.

[00669] 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.

[00670] 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.

[00671] 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.

[00672] 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} , 1×10^{11} to 1×10^{12} , 1×10^{12} to 5×10^{12} , and 5×10^{12} to 1×10^{13} .

[00673] 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.

[00674] 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.

[00675] 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.

IV. Methods of Treating Patients

[00676] 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.

[00677] The expanded TILs produced according the methods described herein, including for example as described in Steps A through I above or according to Steps A through I above (also as shown, for example, in Figure 5) 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.

[00678] 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 G of Figure 5), 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 G of Figure 5), are selected for an additional second expansion according to Step G of Figure 5.

[00679] 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.

[00680] In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 5, 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 5, 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 5. 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 5.

[00681] In some embodiments, IFN- γ (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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. In some embodiments, IFN-gamma (IFN- γ) is indicative of treatment efficacy and/or increased clinical efficacy in the treatment of cancer.

[00682] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 1 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 5. 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 IFN- γ 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 5.

[00683] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 5, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 5, including 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5.

[00684] 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.

[00759] 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.

[00760] 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 5) 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.

[001325] 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 G of Figure 5A and Figure 5C, Step I of Figure 5B, and/or Step H of Figure 5D), 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 G of Figure 5), are selected for an additional second expansion according to Step G of Figure 5.

[001326] 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.

[001327] In some embodiments, the TILs produced by the methods provided herein, for example those exemplified in Figure 5, 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 5, 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 5. 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 5, 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 5.

[001328] 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. In some embodiments, IFN-gamma (IFN- γ) is indicative of treatment efficacy and/or increased clinical efficacy in the treatment of cancer.

[001329] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 5, 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 5. 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 IFN- γ 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 5.

[001330] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 5, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 5, including 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5.

[001331] 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.

A. Methods of Treating Cancers

[001332] The compositions and methods described herein can be used in a method for treating diseases. In some embodiments, they are for use in treating hyperproliferative disorders, such as cancer, in an adult patient or in a pediatric patient. They may also be used in treating other disorders as described herein and in the following paragraphs.

[001333] 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 anal cancer, bladder cancer, breast cancer (including triple-negative breast cancer), bone cancer, cancer caused by human papilloma virus (HPV), central nervous system associated cancer (including ependymoma, medulloblastoma, neuroblastoma, pineoblastoma, and primitive neuroectodermal tumor), cervical cancer (including squamous cell cervical cancer, adenosquamous cervical cancer, and cervical adenocarcinoma), colon cancer, colorectal cancer, endometrial cancer, esophageal cancer, esophagogastric junction cancer, gastric cancer, gastrointestinal cancer, gastrointestinal stromal tumor, glioblastoma,

glioma, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC), hypopharynx cancer, larynx cancer, nasopharynx cancer, oropharynx cancer, and pharynx cancer), kidney cancer, liver cancer, lung cancer (including non-small-cell lung cancer (NSCLC), metastatic NSCLC, and small-cell lung cancer), melanoma (including uveal melanoma, choroidal melanoma, ciliary body melanoma, iris melanoma, or metastatic melanoma), mesothelioma (including malignant pleural mesothelioma), ovarian cancer, pancreatic cancer (including pancreatic ductal adenocarcinoma), penile cancer, rectal cancer, renal cancer, renal cell carcinoma, sarcoma (including Ewing sarcoma, osteosarcoma, rhabdomyosarcoma, and other bone and soft tissue sarcomas), thyroid cancer (including anaplastic thyroid cancer), uterine cancer, and vaginal cancer.

[001334] In some embodiments, the hyperproliferative disorder 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, mantle cell lymphoma, and multiple myeloma. In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the cancer is a hematological malignancy. In some embodiments, the present invention includes a method of treating a patient with a cancer using TILs, MILs, or PBLs modified to downregulate one or more of PD-1, CTLA-4, LAG-3, CISH and CBL-B, wherein the cancer is a hematological malignancy. In some embodiments, the present invention includes a method of treating a patient with a cancer using MILs or PBLs modified to downregulate one or more of PD-1, CTLA-4, LAG-3, CISH and CBL-BRs, wherein the cancer is a hematological malignancy.

[001335] In some embodiments, the cancer is one of the foregoing cancers, including solid tumor cancers and hematological malignancies, that is relapsed or refractory to treatment with at least one prior therapy, including chemotherapy, radiation therapy, or immunotherapy. In some embodiments, the cancer is one of the foregoing cancers that is relapsed or refractory to treatment with at least two prior therapies, including chemotherapy, radiation therapy, and/or immunotherapy. In some embodiments, the cancer is one of the foregoing cancers that is relapsed or refractory to treatment with at least three prior therapies, including chemotherapy, radiation therapy, and/or immunotherapy.

[001336] In some embodiments, the cancer is a microsatellite instability-high (MSI-H) or a mismatch repair deficient (dMMR) cancer. MSI-H and dMMR cancers and testing therefore have been described in Kawakami, *et al.*, *Curr. Treat. Options Oncol.* **2015**, *16*, 30, the disclosures of which are incorporated by reference herein.

[001337] In some embodiments, the present invention includes a method of treating a patient with a cancer using TILs, MILs, or PBLs modified downregulate one or more of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, wherein the patient is a human. In some embodiments, the present invention includes a method of treating a patient with a cancer using TILs, MILs, or PBLs modified to downregulate one or more of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, wherein the patient is a non-human. In some embodiments, the present invention includes a method of treating a patient with a cancer using TILs, MILs, or PBLs modified to downregulate one or more of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, wherein the patient is a companion animal.

[001338] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the cancer is refractory to treatment with a BRAF inhibitor and/or a MEK inhibitor. In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the cancer is refractory to treatment with a BRAF inhibitor selected from the group consisting of vemurafenib, dabrafenib, encorafenib, sorafenib, and pharmaceutically acceptable salts or solvates thereof. In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the cancer is refractory to treatment with a MEK inhibitor selected from the group consisting of trametinib, cobimetinib, binimetinib, selumetinib, pimasertinib, refametinib, and pharmaceutically acceptable salts or solvates thereof. In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the cancer is refractory to treatment with a BRAF inhibitor selected from the group consisting of vemurafenib, dabrafenib, encorafenib, sorafenib, and pharmaceutically acceptable salts or solvates thereof, and a MEK inhibitor selected from the group consisting of trametinib, cobimetinib, binimetinib, selumetinib, pimasertinib, refametinib, and pharmaceutically acceptable salts or solvates thereof.

[001339] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the cancer is a pediatric cancer.

[001340] In some embodiments, the present invention includes a method of treating a patient with a cancer wherein the cancer is uveal melanoma.

[001341] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the uveal melanoma is choroidal melanoma, ciliary body melanoma, or iris melanoma.

[001342] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the pediatric cancer is a neuroblastoma.

[001343] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the pediatric cancer is a sarcoma.

[001344] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the sarcoma is osteosarcoma.

[001345] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the sarcoma is a soft tissue sarcoma.

[001346] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the soft tissue sarcoma is rhabdomyosarcoma, Ewing sarcoma, or primitive neuroectodermal tumor (PNET).

[0005] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the pediatric cancer is a central nervous system (CNS) associated cancer. In some embodiments, the pediatric cancer is refractory to treatment with chemotherapy. In some embodiments, the pediatric cancer is refractory to treatment with radiation therapy. In some embodiments, the pediatric cancer is refractory to treatment with dinutuximab.

[001347] In some embodiments, the present invention includes a method of treating a patient with a cancer, wherein the CNS associated cancer is medulloblastoma, pineoblastoma, glioma, ependymoma, or glioblastoma.

[001348] The compositions and methods described herein can be used in a method for treating cancer, wherein the cancer is refractory or resistant to prior treatment with an anti-PD-1 or anti-PD-L1 antibody. In some embodiments, the patient is a primary refractory patient to an anti-PD-1 or anti-PD-L1 antibody. In some embodiments, the patient shows no prior response to an anti-PD-1 or anti-PD-L1 antibody. In some embodiments, the patient shows a prior response to an

anti-PD-1 or anti-PD-L1 antibody, follow by progression of the patient's cancer. In some embodiments, the cancer is refractory to an anti-CTLA-4 antibody and/or an anti-PD-1 or anti-PD-L1 antibody in combination with at least one chemotherapeutic agent. In some embodiments, the prior chemotherapeutic agent is carboplatin, paclitaxel, pemetrexed, and/or cisplatin. In some prior embodiments, the chemotherapeutic agent(s) is a platinum doublet chemotherapeutic agent. In some embodiments, the platinum doublet therapy comprises a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin and a second chemotherapeutic agent selected from the group consisting of vinorelbine, gemcitabine and a taxane (including for example, paclitaxel, docetaxel or nab-paclitaxel). In some embodiments, the platinum doublet chemotherapeutic agent is in combination with pemetrexed.

[001349] In some embodiments, the NSCLC is PD-L1 negative and/or is from a patient with a cancer that expresses PD-L1 with a tumor proportion score (TPS) of < 1%, as described elsewhere herein.

[001350] In some embodiments, the NSCLC is refractory to a combination therapy comprising an anti-PD-1 or the anti-PD-L1 antibody and a platinum doublet therapy, wherein the platinum doublet therapy comprises:

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

[001351] In some embodiments, the NSCLC is refractory to a combination therapy comprising an anti-PD-1 or the anti-PD-L1 antibody, pemetrexed, and a platinum doublet therapy, wherein the platinum doublet therapy comprises:

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

[001352] In some embodiments, the NSCLC has been treated with an anti-PD-1 antibody. In some embodiments, the NSCLC has been treated with an anti-PD-L1 antibody. In some embodiments, the NSCLC patient is treatment naïve. In some embodiments, the NSCLC has not been treated with an anti-PD-1 antibody. In some embodiments, the NSCLC has not been treated

with an anti-PD-L1 antibody. In some embodiments, the NSCLC has been previously treated with a chemotherapeutic agent. In some embodiments, the NSCLC has been previously treated with a chemotherapeutic agent but is no longer being treated with the chemotherapeutic agent. In some embodiments, the NSCLC patient is anti-PD-1/PD-L1 naïve. In some embodiments, the NSCLC patient has low expression of PD-L1. In some embodiments, the NSCLC patient has treatment naïve NSCLC or is post-chemotherapeutic treatment but anti-PD-1/PD-L1 naïve. In some embodiments, the NSCLC patient is treatment naïve or post-chemotherapeutic treatment but anti-PD-1/PD-L1 naïve and has low expression of PD-L1. In some embodiments, the NSCLC patient has bulky disease at baseline. In some embodiments, the subject has bulky disease at baseline and has low expression of PD-L1. In some embodiments, the NSCLC patient has no detectable expression of PD-L1. In some embodiments, the NSCLC patient is treatment naïve or post-chemotherapeutic treatment but anti-PD-1/PD-L1 naïve and has no detectable expression of PD-L1. In some embodiments, the patient has bulky disease at baseline and has no detectable expression of PD-L1. In some embodiments, the NSCLC patient has treatment naïve NSCLC or post chemotherapy (*e.g.*, post chemotherapeutic agent) but anti-PD-1/PD-L1 naïve who have low expression of PD-L1 and/or have bulky disease at baseline. In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane. In some embodiments, bulky disease is indicated when there are swollen lymph nodes with a short-axis diameter of 20 mm or greater. In some embodiments, the chemotherapeutic includes a standard of care therapeutic for NSCLC.

[001353] In some embodiments, PD-L1 expression is determined by the tumor proportion score. In some embodiments, the subject with a refractory NSCLC tumor has a < 1% tumor proportion score (TPS). In some embodiments, the subject with a refractory NSCLC tumor has a ≥ 1% TPS. In some embodiments, subject with the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and the tumor proportion score was determined prior to said anti-PD-1 and/or anti-PD-L1 antibody treatment. In some embodiments, subject with the refractory NSCLC has been previously treated with an anti-PD-L1 antibody and the tumor proportion score was determined prior to said anti-PD-L1 antibody treatment.

[001354] In some embodiments, the TILs prepared by the methods of the present invention, including those as described for example in Figure 5, exhibit increased polyclonality as compared to TILs produced by other methods, including those not exemplified in Figure 5, 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 5. 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 5. 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 5. 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 5. 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 5. 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 5. 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 5.

[001355] In some embodiments, PD-L1 expression is determined by the tumor proportion score using one more testing methods as described herein. In some embodiments, the subject or patient with a NSCLC tumor has a $< 1\%$ tumor proportion score (TPS). In some embodiments, the NSCLC tumor has a $\geq 1\%$ TPS. In some embodiments, the subject or patient with the NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and the tumor

proportion score was determined prior to the anti-PD-1 and/or anti-PD-L1 antibody treatment. In some embodiments, the subject or patient with the NSCLC has been previously treated with an anti-PD-L1 antibody and the tumor proportion score was determined prior to the anti-PD-L1 antibody treatment. In some embodiments, the subject or patient with a refractory or resistant NSCLC tumor has a $< 1\%$ tumor proportion score (TPS). In some embodiments, the subject or patient with a refractory or resistant NSCLC tumor has a $\geq 1\%$ TPS. In some embodiments, the subject or patient with the refractory or resistant NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and the tumor proportion score was determined prior to the anti-PD-1 and/or anti-PD-L1 antibody treatment. In some embodiments, the subject or patient with the refractory or resistant NSCLC has been previously treated with an anti-PD-L1 antibody and the tumor proportion score was determined prior to the anti-PD-L1 antibody treatment.

[001356] In some embodiments, the NSCLC is an NSCLC that exhibits a tumor proportion score (TPS), or the percentage of viable tumor cells from a patient taken prior to anti-PD-1 or anti-PD-L1 therapy, showing partial or complete membrane staining at any intensity, for the PD-L1 protein that is less than 1% ($TPS < 1\%$). In some embodiments, the NSCLC is an NSCLC that exhibits a TPS selected from the group consisting of $<50\%$, $<45\%$, $<40\%$, $<35\%$, $<30\%$, $<25\%$, $<20\%$, $<15\%$, $<10\%$, $<9\%$, $<8\%$, $<7\%$, $<6\%$, $<5\%$, $<4\%$, $<3\%$, $<2\%$, $<1\%$, $<0.9\%$, $<0.8\%$, $<0.7\%$, $<0.6\%$, $<0.5\%$, $<0.4\%$, $<0.3\%$, $<0.2\%$, $<0.1\%$, $<0.09\%$, $<0.08\%$, $<0.07\%$, $<0.06\%$, $<0.05\%$, $<0.04\%$, $<0.03\%$, $<0.02\%$, and $<0.01\%$. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS selected from the group consisting of about 50%, about 45%, about 40%, about 35%, about 30%, about 25%, about 20%, about 15%, about 10%, about 9%, about 8%, about 7%, about 6%, about 5%, about 4%, about 3%, about 2%, about 1%, about 0.9%, about 0.8%, about 0.7%, about 0.6%, about 0.5%, about 0.4%, about 0.3%, about 0.2%, about 0.1%, about 0.09%, about 0.08%, about 0.07%, about 0.06%, about 0.05%, about 0.04%, about 0.03%, about 0.02%, and about 0.01%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 1%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.9%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.8%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.7%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.6%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.5%. In some embodiments, the NSCLC is an NSCLC that

exhibits a TPS between 0% and 0.4%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.3%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.2%. In some embodiments, the NSCLC is an NSCLC that exhibits a TPS between 0% and 0.1%. TPS may be measured by methods known in the art, such as those described in Hirsch, *et al. J. Thorac. Oncol.* **2017**, *12*, 208-222 or those used for the determination of TPS prior to treatment with pembrolizumab or other anti-PD-1 or anti-PD-L1 therapies. Methods for measurement of TPS that have been approved by the U.S. Food and Drug Administration may also be used. In some embodiments, the PD-L1 is exosomal PD-L1. In some embodiments, the PD-L1 is found on circulating tumor cells.

[001357] In some embodiments, the partial membrane staining includes 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or more. In some embodiments, the completed membrane staining includes approximately 100% membrane staining.

[001358] In some embodiments, testing for PD-L1 can involve measuring levels of PD-L1 in patient serum. In these embodiments, measurement of PD-L1 in patient serum removes the uncertainty of tumor heterogeneity and the patient discomfort of serial biopsies.

[001359] In some embodiments, elevated soluble PD-L1 as compared to a baseline or standard level correlates with worsened prognosis in NSCLC. *See*, for example, Okuma, *et al.*, *Clinical Lung Cancer*, **2018**, *19*, 410-417; Vecchiarelli, *et al.*, *Oncotarget*, **2018**, *9*, 17554-17563. In some embodiments, the PD-L1 is exosomal PD-L1. In some embodiments, the PD-L1 is expressed on circulating tumor cells.

[001360] In some embodiments, the subject or patient has non-small cell lung carcinoma (NSCLC) characterized by at least one of:

- i. a predetermined tumor proportion score (TPS) of PD-L1 < 1%,
- ii. a TPS score of PD-L1 of 1%-49%, or
- iii. a predetermined absence of one or more driver mutations,

wherein the driver mutation is selected from the group consisting of an EGFR mutation, an EGFR insertion, an EGFR exon 20 mutation, a KRAS mutation, a BRAF mutation, an ALK mutation, a c-ROS mutation (ROS1 mutation), a ROS1 fusion, a RET mutation, a RET fusion, an ERBB2 mutation, an

ERBB2 amplification, a BRCA mutation, a MAP2K1 mutation, PIK3CA, CDKN2A, a PTEN mutation, an UMD mutation, an NRAS mutation, a KRAS mutation, an NF1 mutation, a MET mutation, a MET splice and/or altered MET signaling, a TP53 mutation, a CREBBP mutation, a KMT2C mutation, a KMT2D mutation, an ARID1A mutation, a RB1 mutation, an ATM mutation, a SETD2 mutation, a FLT3 mutation, a PTPN11 mutation, a FGFR1 mutation, an EP300 mutation, a MYC mutation, an EZH2 mutation, a JAK2 mutation, a FBXW7 mutation, a CCND3 mutation, and a GNA11 mutation.

[001361] In other embodiments, the invention provides a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the therapeutic TIL population described herein.

[001362] In other embodiments, the invention provides a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the TIL composition described herein.

[001363] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that prior to administering the therapeutically effective dosage of the therapeutic TIL population and the TIL composition described herein, respectively, a non-myeloablative lymphodepletion regimen has been administered to the subject.

[001364] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the non-myeloablative lymphodepletion regimen comprises the steps of 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.

[001365] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified to further comprise the step of treating the subject with a high-dose IL-2 regimen starting on the day after administration of the TIL cells to the subject.

[001366] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the high-dose IL-2 regimen comprises 600,000 or 720,000 IU/kg administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

[001367] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is a solid tumor.

[001368] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, triple negative breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, or renal cell carcinoma.

[001369] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is melanoma, metastatic melanoma, HNSCC, cervical cancers, NSCLC, metastatic NSCLC, glioblastoma (including GBM), and gastrointestinal cancer.

[001370] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is melanoma.

[001371] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is metastatic melanoma.

[001372] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is HNSCC.

[001373] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is a cervical cancer.

[001374] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is NSCLC.

[001375] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is metastatic NSCLC.

[001376] In other embodiments, the invention provides the method for treating a subject with cancer described herein modified such that the cancer is glioblastoma (including GBM).

[001377] In other embodiments, the invention provides a method for treating a subject with cancer described herein modified such that the cancer is gastrointestinal cancer.

[001378] In other embodiments, the invention provides a method for treating a subject with cancer described herein modified such that the cancer is a hypermutated cancer.

[001379] In other embodiments, the invention provides a method for treating a subject with cancer described herein modified such that the cancer is a pediatric hypermutated cancer.

[001380] In other embodiments, the invention provides a therapeutic TIL population described herein for use in a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the therapeutic TIL population.

[001381] In other embodiments, the invention provides a TIL composition described herein for use in a method for treating a subject with cancer comprising administering to the subject a therapeutically effective dosage of the TIL composition.

[001382] In other embodiments, the invention provides a therapeutic TIL population described herein or the TIL composition described herein modified such that prior to administering to the subject the therapeutically effective dosage of the therapeutic TIL population described herein or the TIL composition described herein, a non-myeloablative lymphodepletion regimen has been administered to the subject.

[001383] In other embodiments, the invention provides a therapeutic TIL population or the TIL composition described herein modified such that the non-myeloablative lymphodepletion regimen comprises the steps of 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.

[001384] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified to further comprise the step of treating patient with a high-dose IL-2 regimen starting on the day after administration of the TIL cells to the patient.

[001385] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the high-dose IL-2 regimen comprises 600,000 or 720,000 IU/kg administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

[001386] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is a solid tumor.

[001387] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, triple negative breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, or renal cell carcinoma.

[001388] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is melanoma, metastatic melanoma, HNSCC, cervical cancers, NSCLC, metastatic NSCLC, glioblastoma (including GBM), and gastrointestinal cancer.

[001389] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is melanoma.

[001390] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is metastatic melanoma.

[001391] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is HNSCC.

[001392] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is cervical cancer.

[001393] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is NSCLC.

[001394] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is metastatic NSCLC.

[001395] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is glioblastoma.

[001396] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is gastrointestinal cancer.

[001397] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is a hypermutated cancer.

[001398] In other embodiments, the invention provides a therapeutic TIL population or a TIL composition described herein modified such that the cancer is a pediatric hypermutated cancer.

[001399] In other embodiments, the invention provides the use of a therapeutic TIL population described herein in a method of treating cancer in a subject comprising administering to the subject a therapeutically effective dosage of the therapeutic TIL population.

[001400] In other embodiments, the invention provides the use of a TIL composition described in any of the preceding paragraphs in a method of treating cancer in a subject comprising administering to the subject a therapeutically effective dosage of the TIL composition.

[001401] In other embodiments, the invention provides the use of a therapeutic TIL population described herein or a TIL composition described herein in a method of treating cancer in a patient comprising administering to the patient a non-myeloablative lymphodepletion regimen and then administering to the subject the therapeutically effective dosage of the therapeutic TIL population described in any of the preceding paragraphs or the therapeutically effective dosage of the TIL composition described herein.

1. Combinations with PD-1 and PD-L1 Inhibitors

[001402] In some embodiments, the TIL therapy provided to patients with cancer may include treatment with therapeutic populations of TILs alone or may include a combination treatment including TILs and one or more PD-1 and/or PD-L1 inhibitors.

[001403] 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.

[001404] In some embodiments, 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.

[001405] In some embodiments, 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 some embodiments, 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 some embodiments, the antibody competes for binding with PD-1, and/or binds to an epitope on PD-1.

[001406] In some embodiments, the PD-1 inhibitor is one that binds human PD-1 with a KD of about 100 pM or lower, binds human PD-1 with a KD of about 90 pM or lower, binds human PD-1 with a KD of about 80 pM or lower, binds human PD-1 with a KD of about 70 pM or lower, binds human PD-1 with a KD of about 60 pM or lower, binds human PD-1 with a KD of about 50 pM or lower, binds human PD-1 with a KD of about 40 pM or lower, binds human PD-1 with a KD of about 30 pM or lower, binds human PD-1 with a KD of about 20 pM or lower,

binds human PD-1 with a KD of about 10 pM or lower, or binds human PD-1 with a KD of about 1 pM or lower.

[001407] In some embodiments, the PD-1 inhibitor is one 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.

[001408] In some embodiments, the PD-1 inhibitor is one 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.

[001409] In some embodiments, the PD-1 inhibitor is one that blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 10 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 9 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 8 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 7 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 6 nM or lower, blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ 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.

[001410] In some embodiments, 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 some embodiments, 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 19. 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".

[001411] In some embodiments, a PD-1 inhibitor comprises a heavy chain given by SEQ ID NO:158 and a light chain given by SEQ ID NO:159. In some embodiments, a PD-1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:158 and SEQ ID NO:159, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:158 and SEQ ID NO:159, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:158 and SEQ ID NO:159, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:158 and SEQ ID NO:159, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:158 and SEQ ID NO:159, respectively. In some embodiments, a PD-1 inhibitor

comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:463 and SEQ ID NO:159, respectively.

[001412] In some embodiments, the PD-1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of nivolumab. In some embodiments, the PD-1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:160, and the PD-1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:161, or conservative amino acid substitutions thereof. In some embodiments, 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:160 and SEQ ID NO:161, respectively. In some embodiments, 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:160 and SEQ ID NO:161, respectively. In some embodiments, 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:160 and SEQ ID NO:161, respectively. In some embodiments, 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:160 and SEQ ID NO:161, respectively. In some embodiments, 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:160 and SEQ ID NO:161, respectively.

[001413] In some embodiments, a PD-1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:162, SEQ ID NO:163, and SEQ ID NO:164, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:165, SEQ ID NO:166, and SEQ ID NO:167, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on PD-1 as any of the aforementioned antibodies.

[001414] In some embodiments, the PD-1 inhibitor is an anti-PD-1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to nivolumab. In some embodiments, 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 19. Amino acid sequences for PD-1 inhibitors related to nivolumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO: 158 nivolumab heavy chain	QVQLVESGGG VVQPGKSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRY 60 ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTSLV TSSASTKGPS 120 VFPLAPCSRS TSESTAALGC LVKDYFPEPV TVSWNSGALT SGVHTFPAVL QSSGLYSLSS 180 VVTVPSSSLG TKTYTCNVDH KPSNTKVDKR VESKYGPPCP PCPAPEFLGG PSVFLFPPKP 240 KDTLMISRTPEVTCVVVDVSDQEDPEVFQFNW YVDGVEVHNA KTKPREEQFN STYRVVSVLT 300 VLHQDWLNGK EYKCKVSNKGLPSSIEK TIS KAKGQPREPQ VYTLPPSQEE MTKNQVSLT 360 LVKGFYPSDI AVEWESNGQP ENNYKTTTPV LDSGGSFFLY SRLTVDKSRW QEGNVFSCSV 420 MHEALHNHYT QKSLSLGLK 440
SEQ ID NO: 159 nivolumab light chain	EIVLTQSPATLSLSPGERAT LSCRASQSVS SYLAWYQQKPGQAPRLLIYD ASNRATGIPA 60 RFGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIKRTV AAPSVFIFPP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGE 214
SEQ ID NO: 160 nivolumab variable heavy chain	QVQLVESGGG VVQPGKSLRL DCKASGITFS NSGMHWVRQA PGKGLEWVAV IWYDGSKRY 60 ADSVKGRFTI SRDNSKNTLF LQMNSLRAED TAVYYCATND DYWGQGTSLV TSS 113
SEQ ID NO: 161 nivolumab variable light chain	EIVLTQSPATLSLSPGERAT LSCRASQSVS SYLAWYQQKPGQAPRLLIYD ASNRATGIPA 60 RFGSGSGTD FTLTISSLEP EDFAVYYCQQ SSNWPRTFGQ GTKVEIK 107
SEQ ID NO: 162 nivolumab heavy chain CDR1	NSGMH 5
SEQ ID NO: 163 nivolumab heavy chain CDR2	VIWYDGSKRY YADSVKG 17
SEQ ID NO: 164 nivolumab heavy chain CDR3	NDDY 4

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO: 165 nivolumab light chain CDR1	RASQSVSSYL A 11
SEQ ID NO: 166 nivolumab light chain CDR2	DASNRAT 7
SEQ ID NO: 167 nivolumab light chain CDR3	QQSSNWPRT 9

[001415] In some embodiments, the PD-1 inhibitor is nivolumab or a biosimilar thereof, and the nivolumab is administered at a dose of about 0.5 mg/kg to about 10 mg/kg. In some embodiments, the PD-1 inhibitor is nivolumab or a biosimilar thereof, and the nivolumab is administered at a dose of about 0.5 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 2.5 mg/kg, about 3 mg/kg, about 3.5 mg/kg, about 4 mg/kg, about 4.5 mg/kg, about 5 mg/kg, about 5.5 mg/kg, about 6 mg/kg, about 6.5 mg/kg, about 7 mg/kg, about 7.5 mg/kg, about 8 mg/kg, about 8.5 mg/kg, about 9 mg/kg, about 9.5 mg/kg, or about 10 mg/kg. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001416] In some embodiments, the PD-1 inhibitor is nivolumab or a biosimilar thereof, and the nivolumab is administered at a dose of about 200 mg to about 500 mg. In some embodiments, the PD-1 inhibitor is nivolumab or a biosimilar thereof, and the nivolumab is administered at a dose of about 200 mg, about 220 mg, about 240 mg, about 260 mg, about 280 mg, about 300 mg, about 320 mg, about 340 mg, about 360 mg, about 380 mg, about 400 mg, about 420 mg, about 440 mg, about 460 mg, about 480 mg, or about 500 mg. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001417] In some embodiments, the PD-1 inhibitor is nivolumab or a biosimilar thereof, and the nivolumab is administered every 2 weeks, every 3 weeks, every 4 weeks, every 5 weeks, or every 6 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001418] In some embodiments, the nivolumab is administered to treat unresectable or metastatic melanoma. In some embodiments, the nivolumab is administered to treat unresectable or metastatic melanoma and is administered at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat unresectable or metastatic melanoma and is administered at about 480 mg every 4 weeks. In some embodiments, the nivolumab is administered to treat unresectable or metastatic melanoma and is administered at about 1 mg/kg followed by ipilimumab 3 mg/kg on the same day every 3 weeks for 4 doses, then 240 mg every 2 weeks or 480 mg every 4 weeks.

[001419] In some embodiments, the nivolumab is administered for the adjuvant treatment of melanoma. In some embodiments, the nivolumab is administered for the adjuvant treatment of melanoma at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered for the adjuvant treatment of melanoma at about 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001420] In some embodiments, the nivolumab is administered to treat metastatic non-small cell lung cancer. In some embodiments, the nivolumab is administered to treat metastatic non-small cell lung cancer at about 3 mg/kg every 2 weeks along with ipilimumab at about 1 mg/kg every 6 weeks. In some embodiments, the nivolumab is administered to treat metastatic

non-small cell lung cancer at about 360 mg every 3 weeks with ipilimumab 1 mg/kg every 6 weeks and 2 cycles of platinum-doublet chemotherapy. In some embodiments, the nivolumab is administered to treat metastatic non-small cell lung cancer at about 240 mg every 2 weeks or 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001421] In some embodiments, the nivolumab is administered to treat small cell lung cancer. In some embodiments, the nivolumab is administered to treat small cell lung cancer at about 240 mg every 2 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001422] In some embodiments, the nivolumab is administered to treat malignant pleural mesothelioma at about 360 mg every 3 weeks with ipilimumab 1 mg/kg every 6 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001423] In some embodiments, the nivolumab is administered to treat advanced renal cell carcinoma. In some embodiments, the nivolumab is administered to treat advanced renal cell carcinoma at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat advanced renal cell carcinoma at about 480 mg every 4 weeks. In some embodiments, the nivolumab is administered to treat advanced renal cell carcinoma at about 3 mg/kg followed by

ipilimumab at about 1 mg/kg on the same day every 3 weeks for 4 doses, then 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat advanced renal cell carcinoma at about 3 mg/kg followed by ipilimumab at about 1 mg/kg on the same day every 3 weeks for 4 doses, then 240 mg every 2 weeks 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001424] In some embodiments, the nivolumab is administered to treat classical Hodgkin lymphoma. In some embodiments, the nivolumab is administered to treat classical Hodgkin lymphoma at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat classical Hodgkin lymphoma at about 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001425] In some embodiments, the nivolumab is administered to treat Recurrent or metastatic squamous cell carcinoma of the head and neck. In some embodiments, the nivolumab is administered to treat recurrent or metastatic squamous cell carcinoma of the head and neck at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat recurrent or metastatic squamous cell carcinoma of the head and neck at about 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001426] In some embodiments, the nivolumab is administered to treat locally advanced or metastatic urothelial carcinoma at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat locally advanced or metastatic urothelial carcinoma at about 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001427] In some embodiments, the nivolumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer. In some embodiments, the nivolumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer in adult and pediatric patients. In some embodiments, the nivolumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer in adult and pediatric patients ≥ 40 kg at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer in adult and pediatric patients ≥ 40 kg at about 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001428] In some embodiments, the nivolumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer in pediatric patients < 40 kg at about 3 mg/kg every 2 weeks. In some embodiments, the nivolumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer in adult and pediatric patients ≥ 40 kg at about 3 mg/kg followed by ipilimumab 1 mg/kg on the same day every 3 weeks for 4 doses, then 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat microsatellite instability-

high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer in adult and pediatric patients ≥ 40 kg at about 3 mg/kg followed by ipilimumab 1 mg/kg on the same day every 3 weeks for 4 doses, then 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001429] In some embodiments, the nivolumab is administered to treat hepatocellular carcinoma. In some embodiments, the nivolumab is administered to treat hepatocellular carcinoma at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat hepatocellular carcinoma at about 480 mg every 4 weeks. In some embodiments, the nivolumab is administered to treat hepatocellular carcinoma at about 1 mg/kg followed by ipilimumab 3 mg/kg on the same day every 3 weeks for 4 doses, then 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat hepatocellular carcinoma at about 1 mg/kg followed by ipilimumab 3 mg/kg on the same day every 3 weeks for 4 doses, then 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001430] In some embodiments, the nivolumab is administered to treat esophageal squamous cell carcinoma. In some embodiments, the nivolumab is administered to treat esophageal squamous cell carcinoma at about 240 mg every 2 weeks. In some embodiments, the nivolumab is administered to treat esophageal squamous cell carcinoma at about 480 mg every 4 weeks. In some embodiments, the nivolumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the nivolumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the nivolumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In

some embodiments, the nivolumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001431] In other embodiments, 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 20. 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).

[001432] In some embodiments, a PD-1 inhibitor comprises a heavy chain given by SEQ ID NO:168 and a light chain given by SEQ ID NO:169. In some embodiments, a PD-1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:168 and SEQ ID

NO:169, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:168 and SEQ ID NO:169, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:168 and SEQ ID NO:169, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:168 and SEQ ID NO:169, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:168 and SEQ ID NO:169, respectively. In some embodiments, a PD-1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:168 and SEQ ID NO:169, respectively.

[001433] In some embodiments, the PD-1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of pembrolizumab. In some embodiments, the PD-1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:170, and the PD-1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:171, or conservative amino acid substitutions thereof. In some embodiments, 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:170 and SEQ ID NO:171, respectively. In some embodiments, 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:170 and SEQ ID NO:171, respectively. In some embodiments, 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:170 and SEQ ID NO:171, respectively. In some embodiments, 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:170 and SEQ ID NO:171, respectively. In some embodiments, 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:170 and SEQ ID NO:171, respectively.

[001434] In some embodiments, a PD-1 inhibitor comprises the heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:172, SEQ ID NO:173, and SEQ ID NO:174, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:175, SEQ ID NO:176,

and SEQ ID NO:177, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on PD-1 as any of the aforementioned antibodies.

[001435] In some embodiments, the PD-1 inhibitor is an anti-PD-1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to pembrolizumab. In some embodiments, 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 20. Amino acid sequences for PD-1 inhibitors related to pembrolizumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:168 pembrolizumab heavy chain	QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF 60 NEKFKNRVTL TIDSSITTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTTVVSS 120 ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS 180 GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPPCP APEFLGGPSV 240

Identifier	Sequence (One-Letter Amino Acid Symbols)
	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: 169 pembrolizumab light chain	EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES 60 GVPARFSGSG SGTDFLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI KRTVAAPSVF 120 IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSL 180 STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC 218
SEQ ID NO: 170 pembrolizumab variable heavy chain	QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYMYWVRQA PGQGLEWMGG INPSNGGTNF 60 NEKFKNRVTL TDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTTVTSS 120
SEQ ID NO: 171 pembrolizumab variable light chain	EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES 60 GVPARFSGSG SGTDFLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI K 111
SEQ ID NO: 172 pembrolizumab heavy chain CDR1	NYMY 5
SEQ ID NO: 173 pembrolizumab heavy chain CDR2	GINPSNGGTN FNEKFK 16
SEQ ID NO: 174 pembrolizumab heavy chain CDR3	RDYRFDMGFD Y 11
SEQ ID NO: 175 pembrolizumab light chain CDR1	RASKGVSTSG YSYLH 15
SEQ ID NO: 176 pembrolizumab light chain CDR2	LASYLES 7
SEQ ID NO: 177 pembrolizumab light chain CDR3	QHSRDLPLT 9

[001436] In some embodiments, the PD-1 inhibitor is pembrolizumab or a biosimilar thereof, and the pembrolizumab is administered at a dose of about 0.5 mg/kg to about 10 mg/kg. In some embodiments, the PD-1 inhibitor is pembrolizumab or a biosimilar thereof, and the pembrolizumab is administered at a dose of about 0.5 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 2.5 mg/kg, about 3 mg/kg, about 3.5 mg/kg, about 4 mg/kg, about 4.5 mg/kg, about 5 mg/kg, about 5.5 mg/kg, about 6 mg/kg, about 6.5 mg/kg, about 7 mg/kg, about 7.5 mg/kg, about 8 mg/kg, about 8.5 mg/kg, about 9 mg/kg, about 9.5 mg/kg, or about 10 mg/kg. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001437] In some embodiments, the PD-1 inhibitor is pembrolizumab or a biosimilar thereof, wherein the pembrolizumab is administered at a dose of about 200 mg to about 500 mg. In some embodiments, the PD-1 inhibitor is pembrolizumab or a biosimilar thereof, and the nivolumab is administered at a dose of about 200 mg, about 220 mg, about 240 mg, about 260 mg, about 280 mg, about 300 mg, about 320 mg, about 340 mg, about 360 mg, about 380 mg, about 400 mg, about 420 mg, about 440 mg, about 460 mg, about 480 mg, or about 500 mg. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001438] In some embodiments, the PD-1 inhibitor is pembrolizumab or a biosimilar thereof, wherein the pembrolizumab is administered every 2 weeks, every 3 weeks, every 4 weeks, every 5 weeks, or every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001439] In some embodiments, the pembrolizumab is administered to treat melanoma. In some embodiments, the pembrolizumab is administered to treat melanoma at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat melanoma at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001440] In some embodiments, the pembrolizumab is administered to treat NSCLC. In some embodiments, the pembrolizumab is administered to treat NSCLC at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat NSCLC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001441] In some embodiments, the pembrolizumab is administered to treat small cell lung cancer (SCLC). In some embodiments, the pembrolizumab is administered to treat SCLC at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat SCLC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001442] In some embodiments, the pembrolizumab is administered to treat head and neck squamous cell cancer (HNSCC). In some embodiments, the pembrolizumab is administered to treat HNSCC at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat HNSCC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001443] In some embodiments, the pembrolizumab is administered to treat classical Hodgkin lymphoma (cHL) or primary mediastinal large B-cell lymphoma (PMBCL) at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat classical Hodgkin lymphoma (cHL) or primary mediastinal large B-cell lymphoma (PMBCL) at about 400 mg every 6 weeks for adults. In some embodiments, the pembrolizumab is administered to treat classical Hodgkin lymphoma (cHL) or primary mediastinal large B-cell lymphoma (PMBCL) at about 2 mg/kg (up to 200 mg) every 3 weeks for pediatrics. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001444] In some embodiments, the pembrolizumab is administered to treat urothelial carcinoma at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat urothelial carcinoma at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001445] In some embodiments, the pembrolizumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) cancer at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat MSI-H or dMMR cancer at about 400 mg every 6 weeks for adults. In some embodiments, the pembrolizumab is administered to treat MSI-H or dMMR cancer at about 2 mg/kg (up to 200 mg) every 3 weeks for pediatrics. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample

from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001446] In some embodiments, the pembrolizumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient colorectal cancer (dMMR CRC) at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat MSI-H or dMMR CRC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001447] In some embodiments, the pembrolizumab is administered to treat gastric cancer at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat gastric cancer at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001448] In some embodiments, the pembrolizumab is administered to treat Esophageal Cancer at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat Esophageal Cancer at about 400 mg every 6 weeks. In some embodiments, the

pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001449] In some embodiments, the pembrolizumab is administered to treat cervical cancer at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat cervical cancer at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001450] In some embodiments, the pembrolizumab is administered to treat hepatocellular carcinoma (HCC) at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat HCC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001451] In some embodiments, the pembrolizumab is administered to treat Merkel cell carcinoma (MCC) at about 200 mg every 3 weeks for adults. In some embodiments, the pembrolizumab is administered to treat MCC at about 400 mg every 6 weeks for adults. In some embodiments, the pembrolizumab is administered to treat MCC at about 2 mg/kg (up to 200 mg) every 3 weeks for pediatrics. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab

administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001452] In some embodiments, the pembrolizumab is administered to treat renal cell carcinoma (RCC) at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat RCC at about 400 mg every 6 weeks with axitinib 5 mg orally twice daily. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001453] In some embodiments, the pembrolizumab is administered to treat endometrial carcinoma at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat endometrial carcinoma at about 400 mg every 6 weeks with lenvatinib 20 mg orally once daily for tumors that are not MSI-H or dMMR. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001454] In some embodiments, the pembrolizumab is administered to treat tumor mutational burden-high (TMB-H) Cancer at about 200 mg every 3 weeks for adults. In some embodiments, the pembrolizumab is administered to treat TMB-H Cancer at about 400 mg every 6 weeks for adults. In some embodiments, the pembrolizumab is administered to treat TMB-H Cancer at about 2 mg/kg (up to 200 mg) every 3 weeks for pediatrics. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001455] In some embodiments, the pembrolizumab is administered to treat cutaneous squamous cell carcinoma (cSCC) at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat cSCC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001456] In some embodiments, the pembrolizumab is administered to treat triple-negative breast cancer (TNBC) at about 200 mg every 3 weeks. In some embodiments, the pembrolizumab is administered to treat TNBC at about 400 mg every 6 weeks. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001457] In some embodiments, if the patient or subject is an adult, *i.e.*, treatment of adult indications, and additional dosing regimen of 400 mg every 6 weeks can be employed. In some embodiments, the pembrolizumab administration is begun 1, 2, 3, 4, or 5 days post IL-2 administration. In some embodiments, the pembrolizumab administration is begun 1, 2, or 3 days post IL-2 administration. In some embodiments, the pembrolizumab can also be administered 1, 2, 3, 4 or 5 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient). In some embodiments, the pembrolizumab can also be administered 1, 2, or 3 weeks pre-resection (*i.e.*, before obtaining a tumor sample from the subject or patient).

[001458] In some embodiments, 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.

[001459] In some embodiments, 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 some embodiments, 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 other embodiments, 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 some embodiments, 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.

[001460] In some embodiments, 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. In some embodiments, the PD-1 inhibitor is cemiplimab, which is commercially available from Regeneron, Inc.

[001461] In some embodiments, 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.

[001462] 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 some embodiments, 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 some embodiments, 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 some embodiments, the antibody competes for binding with PD-L1 or PD-L2, and/or binds to an epitope on PD-L1 or PD-L2.

[001463] 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.

[001464] 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.

[001465] 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 some embodiments, the tumor cells express PD-L1. In other embodiments, the tumor cells do not express PD-L1. In some embodiments, the methods can include a combination of a PD-1 and a PD-L1 antibody, such as those described herein, in combination with a TIL. The administration of a combination of a PD-1 and a PD-L1 antibody and a TIL may be simultaneous or sequential.

[001466] In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that binds human PD-L1 and/or PD-L2 with a K_D of about 100 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 90 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 80 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 70 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 60 pM or lower, a K_D of about 50 pM or lower, binds human PD-L1 and/or PD-L2 with a K_D of about 40 pM or lower, or binds human PD-L1 and/or PD-L2 with a K_D of about 30 pM or lower,

[001467] In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that binds to human PD-L1 and/or PD-L2 with a k_{assoc} of about 7.5×10^5 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 1×10^6 1/M·s or faster.

[001468] In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that binds to human PD-L1 or PD-L2 with a k_{dissoc} of about 2×10^{-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.

[001469] In some embodiments, the PD-L1 and/or PD-L2 inhibitor is one that blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 10 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 9 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 8 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 7 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 6 nM or lower; blocks or inhibits binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ 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 human PD-1, or blocks binding of human PD-L1 or human PD-L2 to human PD-1 with an IC₅₀ of about 1 nM or lower.

[001470] In some embodiments, 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 some embodiments, 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 21. 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".

[001471] In some embodiments, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:178 and a light chain given by SEQ ID NO:179. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:178 and SEQ ID NO:179, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:178 and SEQ ID NO:179, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:178 and SEQ ID NO:179, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:178 and SEQ ID NO:179, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:178 and SEQ ID NO:179, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:178 and SEQ ID NO:179, respectively.

[001472] In some embodiments, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of durvalumab. In some embodiments, the PD-L1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:180, and the PD-L1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:181, or conservative amino acid substitutions thereof. In some embodiments, 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:180 and SEQ ID NO:181, respectively. In some embodiments, 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:180 and SEQ ID NO:181, respectively. In some embodiments, 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:180 and SEQ ID NO:181, respectively. In some embodiments, 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:180 and SEQ ID NO:181, respectively. In some embodiments, 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:180 and SEQ ID NO:181, respectively.

[001473] In some embodiments, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:182, SEQ ID NO:183, and SEQ ID NO:184, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:185, SEQ ID NO:186, and SEQ ID NO:187, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

[001474] In some embodiments, the PD-L1 inhibitor is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to durvalumab. In some embodiments, 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 21. Amino acid sequences for PD-L1 inhibitors related to durvalumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO: 178 durvalumab heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN IKQDGSEKYY 60 VDSVKGRFTI SRDPAKNSLY LQMNSLRAED TAVYYCAREG GWFGELAFDY WQGTGLTVTS 120 SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS 180 SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVK PKSCDKTHTC PPCPAPEFEG 240 GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY 300 NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPASIEKTI SKAKGQPREP QVYTLPPSRE 360 EMTKNQVSLT CLVKGFYPSD IAEWEWSNGQ PENNYKTIPP VLDSGDSFFL YSKLTVDKSR 420 WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 451
SEQ ID NO: 179 durvalumab light chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN EIVLTQSPGT 60 LSLSPGERAT LSCRASQKVS SSYLAWYQQK PGQAPRLLIY DASSRATGIP DRFSGSGSGT 120 DFTLTISRLE PEDFAVYYCQ QYGSLPWTFG QGTEVEIKRT VAAPSVFIFP PSDEQLKSGT 180 ASVVCLLNMF YPREAKVQWK VDNALQSGNS QESVTEQDSK DSTYSLSTL TSKADYEKH 240 KVYACEVTHQ GLSSPVTKSF NRGEK 265
SEQ ID NO: 180 durvalumab variable heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS RYWMSWVRQA PGKGLEWVAN IKQDGSEKYY 60 VDSVKGRFTI SRDPAKNSLY LQMNSLRAED TAVYYCAREG GWFGELAFDY WQGTGLTVTS 120 S 121
SEQ ID NO: 181 durvalumab variable light chain	EIVLTQSPGT LSLSPGERAT LSCRASQKVS SSYLAWYQQK PGQAPRLLIY DASSRATGIP 60 DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSLPWTFG QGTEVEIK 108
SEQ ID NO: 182 durvalumab heavy chain CDR1	RYWMS 5
SEQ ID NO: 183 durvalumab heavy chain CDR2	NIKQDGSEKY YVDSVKG 17
SEQ ID NO: 184 durvalumab heavy chain CDR3	EGGWFGELAF DY 12
SEQ ID NO: 185 durvalumab light chain CDR1	RASQRVSSSY LA 12
SEQ ID NO: 186 durvalumab light chain CDR2	DASSRAT 7
SEQ ID NO: 187 durvalumab light chain CDR3	QYGSLPWT 9

[001475] In some embodiments, 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 22. 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'.

[001476] In some embodiments, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:188 and a light chain given by SEQ ID NO:189. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:188 and SEQ ID NO:189, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:188 and SEQ ID NO:189, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:188 and SEQ ID NO:189, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:188 and SEQ ID NO:189, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:188 and SEQ ID NO:189, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:188 and SEQ ID NO:189, respectively.

[001477] In some embodiments, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of avelumab. In some embodiments, the PD-L1 inhibitor heavy chain variable region (VH) comprises the sequence shown in SEQ ID NO:190, and the PD-L1 inhibitor light chain variable region (VL) comprises the sequence shown in SEQ ID NO:191, or conservative amino acid substitutions thereof. In some embodiments, 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:190 and SEQ ID NO:191, respectively. In some embodiments, 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:190 and SEQ ID NO:191, respectively. In some embodiments, 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:190 and SEQ ID NO:191, respectively. In some embodiments, 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:190 and SEQ ID NO:191, respectively. In some embodiments, 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:190 and SEQ ID NO:191, respectively.

[001478] In some embodiments, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:192, SEQ ID NO:193, and SEQ ID NO:194, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:195, SEQ ID NO:196, and SEQ ID NO:197, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

[001479] In some embodiments, the PD-L1 inhibitor is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to avelumab. In some embodiments, the biosimilar comprises an anti-PD-L1 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is an anti-PD-L1 antibody authorized or submitted for authorization, wherein the anti-PD-L1 antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is avelumab. The anti-PD-L1 antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or

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

TABLE 22. Amino acid sequences for PD-L1 inhibitors related to avelumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO: 188 avelumab heavy chain	EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS IYPSGGITFY 60 ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK LGTVTTVDYW GQGTLVTVSS 120 ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTEPAVLQSS 180 GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG 240 PSVFLFPPKP KDTLMISRTI EPTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN 300 STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEK TIS KAKGQPREPQ VYTLPPSRDE 360 LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTTPV LDSDGSFFLY SKLTVDKSRW 420 QQGNVFSCSV MHEALHNNHYT QKSLSLSPGK 450
SEQ ID NO: 189 avelumab light chain	QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLMY YDVSNNRPSGV 60 SNRFGSKSG NTASLTISGL QAEDADYYC SSYTSSSTRV FGTGKVTVL GQPKANPTVT 120 LFPPSSEELQ ANKATLVCLI SDFYPGAVTV AWKADGSPVK AGVETTKPSK QSNKNYAASS 180 YLSLTPEQWK SHRSYSCQVT HEGSTVEKTV APTECS 216
SEQ ID NO: 190 avelumab variable heavy chain	EVQLLESGGG LVQPGGSLRL SCAASGFTFS SYIMMWVRQA PGKGLEWVSS IYPSGGITFY 60 ADTVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARIK LGTVTTVDYW GQGTLVTVSS 120
SEQ ID NO: 191 avelumab variable light chain	QSALTQPASV SGSPGQSITI SCTGTSSDVG GYNYVSWYQQ HPGKAPKLMY YDVSNNRPSGV 60 SNRFGSKSG NTASLTISGL QAEDADYYC SSYTSSSTRV FGTGKVTVL 110
SEQ ID NO: 192 avelumab heavy chain CDR1	SYIMM 5
SEQ ID NO: 193 avelumab heavy chain CDR2	SIYPSGGITF YADTVKG 17
SEQ ID NO: 194 avelumab heavy chain CDR3	IKLGTVTIVD Y 11
SEQ ID NO: 195 avelumab light chain CDR1	TGTSSDVG Y NYVS 14
SEQ ID NO: 196 avelumab light chain CDR2	DVSNRPS 7
SEQ ID NO: 197 avelumab light chain CDR3	SSYTSSSTRV 10

[001480] In some embodiments, 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 some embodiments, 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

some embodiments, 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 23. 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'.

[001481] In some embodiments, a PD-L1 inhibitor comprises a heavy chain given by SEQ ID NO:198 and a light chain given by SEQ ID NO:199. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:198 and SEQ ID NO:199, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:198 and SEQ ID NO:199, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:198 and SEQ ID NO:199, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:198 and SEQ ID NO:199, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:198 and SEQ ID NO:199, respectively. In some embodiments, a PD-L1 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:198 and SEQ ID NO:199, respectively.

[001482] In some embodiments, the PD-L1 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of atezolizumab. In some embodiments, the PD-L1 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:200, and the PD-L1 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:201, or conservative amino acid substitutions thereof. In some embodiments, 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:200 and SEQ ID NO:201, respectively. In some embodiments, 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:200 and SEQ ID NO:201, respectively. In some embodiments, 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:200 and SEQ ID NO:201, respectively. In some embodiments, 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:200 and SEQ ID NO:201, respectively. In some embodiments, 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:200 and SEQ ID NO:201, respectively.

[001483] In some embodiments, a PD-L1 inhibitor comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:202, SEQ ID NO:203, and SEQ ID NO:204, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:205, SEQ ID NO:206, and SEQ ID NO:207, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on PD-L1 as any of the aforementioned antibodies.

[001484] In some embodiments, the anti-PD-L1 antibody is an anti-PD-L1 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to atezolizumab. In some embodiments, 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 23. Amino acid sequences for PD-L1 inhibitors related to atezolizumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:198 atezolizumab heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS DSWIHWVRQA PGKGLEWVAV ISPYGGSTYY 60 ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCARRH WPGGFDYWGQ GTLTVTVSSAS 120 TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPTVSWN SGALTSGVHT FPAVLQSSGL 180 YSLSSVTVTP SSSLGTQTYI CNVNHKPSNT KVDKKVEPKS CDKTHTCPPC PAPELLGGPS 240 VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV DGVEVHNAKT KPREEQYAST 300 YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT 360 KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ 420 GNVFSCSVMH EALHNHYTQK SLSLSPGK 448
SEQ ID NO:199 atezolizumab light chain	DIQMTQSPSS LSASVGDRVT ITCRASQDVS TAVAWYQQKPK GKAPKLLIYS ASFLYSGVPS 60 RFGSGSGGTD FTLTISSLQP EDFATYYCQQ YLYHPATFGQ GTKVEIKRTV AAPSVFIFPP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSLSTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEFC 214
SEQ ID NO:200 atezolizumab variable heavy chain	EVQLVESGGG LVQPGGSLRL SCAASGFTFS DSWIHWVRQA PGKGLEWVAV ISPYGGSTYY 60 ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCARRH WPGGFDYWGQ GTLTVTVSA 118
SEQ ID NO:201 atezolizumab variable light chain	DIQMTQSPSS LSASVGDRVT ITCRASQDVS TAVAWYQQKPK GKAPKLLIYS ASFLYSGVPS 60 RFGSGSGGTD FTLTISSLQP EDFATYYCQQ YLYHPATFGQ GTKVEIKR 108
SEQ ID NO:202 atezolizumab heavy chain CDR1	GFTFSDSWIH 10
SEQ ID NO:203 atezolizumab heavy chain CDR2	AWISPYGGST YYADSVKG 18
SEQ ID NO:204 atezolizumab heavy chain CDR3	RHWPGGFDY 9
SEQ ID NO:205 atezolizumab light chain CDR1	RASQDVSTAV A 11
SEQ ID NO:206 atezolizumab light chain CDR2	SASFLYS 7
SEQ ID NO:207 atezolizumab light chain CDR3	QQYLYHPAT 9

[001485] In some embodiments, PD-L1 inhibitors include those antibodies described in U.S. Patent Application Publication No. US 2014/0341917 A1, the disclosure of which is incorporated by reference herein. In other embodiments, antibodies that compete with any of

these antibodies for binding to PD-L1 are also included. In some embodiments, the anti-PD-L1 antibody is MDX-1105, also known as BMS-935559, which is disclosed in U.S. Patent No. US 7,943,743, the disclosures of which are incorporated by reference herein. In some embodiments, the anti-PD-L1 antibody is selected from the anti-PD-L1 antibodies disclosed in U.S. Patent No. US 7,943,743, which are incorporated by reference herein.

[001486] In some embodiments, the PD-L1 inhibitor is a commercially-available monoclonal antibody, such as INVIVOMAB anti-m-PD-L1 clone 10F.9G2 (Catalog # BE0101, Bio X Cell, Inc., West Lebanon, NH, USA). In some embodiments, the anti-PD-L1 antibody is a commercially-available monoclonal antibody, such as AFFYMETRIX EBIOSCIENCE (MIH1). A number of commercially-available anti-PD-L1 antibodies are known to one of ordinary skill in the art.

[001487] In some embodiments, the PD-L2 inhibitor is a commercially-available monoclonal antibody, such as BIOLEGEND 24F.10C12 Mouse IgG2a, κ isotype (catalog # 329602 Biologend, Inc., San Diego, CA), SIGMA anti-PD-L2 antibody (catalog # SAB3500395, Sigma-Aldrich Co., St. Louis, MO), or other commercially-available anti-PD-L2 antibodies known to one of ordinary skill in the art.

2. Combinations with CTLA-4 Inhibitors

[001488] In some embodiments, the TIL therapy provided to patients with cancer may include treatment with therapeutic populations of TILs alone or may include a combination treatment including TILs and one or more CTLA-4 inhibitors.

[001489] Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a member of the immunoglobulin superfamily and is expressed on the surface of helper T cells. CTLA-4 is a negative regulator of CD28-dependent T cell activation and acts as a checkpoint for adaptive immune responses. Similar to the T cell costimulatory protein CD28, the CTLA-4 binding antigen presents CD80 and CD86 on the cells. CTLA-4 delivers a suppressor signal to T cells, while CD28 delivers a stimulus signal. Human antibodies against human CTLA-4 have been described as immunostimulatory modulators in many disease conditions, such as treating or preventing viral and bacterial infections and for treating cancer (WO 01/14424 and WO 00/37504). A number of fully human anti-human CTLA-4 monoclonal antibodies (mAbs) have been studied in clinical

trials for the treatment of various types of solid tumors, including, but not limited to, ipilimumab (MDX-010) and tremelimumab (CP-675,206).

[001490] In some embodiments, a CTLA-4 inhibitor may be any CTLA-4 inhibitor or CTLA-4 blocker known in the art. In particular, it is one of the CTLA-4 inhibitors or blockers described in more detail in the following paragraphs. The terms “inhibitor,” “antagonist,” and “blocker” are used interchangeably herein in reference to CTLA-4 inhibitors. For avoidance of doubt, references herein to a CTLA-4 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 CTLA-4 inhibitor may also refer to a small molecule compound or a pharmaceutically acceptable salt, ester, solvate, hydrate, cocrystal, or prodrug thereof.

[001491] Suitable CTLA-4 inhibitors for use in the methods of the invention, include, without limitation, anti-CTLA-4 antibodies, human anti-CTLA-4 antibodies, mouse anti-CTLA-4 antibodies, mammalian anti-CTLA-4 antibodies, humanized anti-CTLA-4 antibodies, monoclonal anti-CTLA-4 antibodies, polyclonal anti-CTLA-4 antibodies, chimeric anti-CTLA-4 antibodies, MDX-010 (ipilimumab), tremelimumab, anti-CD28 antibodies, anti-CTLA-4 adnectins, anti-CTLA-4 domain antibodies, single chain anti-CTLA-4 fragments, heavy chain anti-CTLA-4 fragments, light chain anti-CTLA-4 fragments, inhibitors of CTLA-4 that agonize the co-stimulatory pathway, the antibodies disclosed in PCT Publication No. WO 2001/014424, the antibodies disclosed in PCT Publication No. WO 2004/035607, the antibodies disclosed in U.S. Publication No. 2005/0201994, and the antibodies disclosed in granted European Patent No. EP 1212422 B1, the disclosures of each of which are incorporated herein by reference. Additional CTLA-4 antibodies are described in U.S. Pat. Nos. 5,811,097, 5,855,887, 6,051,227, and 6,984,720; in PCT Publication Nos. WO 01/14424 and WO 00/37504; and in U.S. Publication Nos. 2002/0039581 and 2002/086014, the disclosures of each of which are incorporated herein by reference. Other anti-CTLA-4 antibodies that can be used in a method of the present invention include, for example, those disclosed in: WO 98/42752; U.S. Pat. Nos. 6,682,736 and 6,207,156; Hurwitz *et al.*, Proc. Natl. Acad. Sci. USA, 95(17):10067-10071 (1998); Camacho *et al.*, J. Clin. Oncology, 22(145): Abstract No. 2505 (2004) (antibody CP-675206); Mokyr *et al.*, Cancer Res., 58:5301-5304 (1998), and U.S. Pat. Nos. 5,977,318, 6,682,736, 7,109,003, and 7,132,281, the disclosures of each of which are incorporated herein by reference.

[001492] Additional CTLA-4 inhibitors include, but are not limited to, the following: any inhibitor that is capable of disrupting the ability of CD28 antigen to bind to its cognate ligand, to inhibit the ability of CTLA-4 to bind to its cognate ligand, to augment T cell responses via the co-stimulatory pathway, to disrupt the ability of B7 to bind to CD28 and/or CTLA-4, to disrupt the ability of B7 to activate the co-stimulatory pathway, to disrupt the ability of CD80 to bind to CD28 and/or CTLA-4, to disrupt the ability of CD80 to activate the co-stimulatory pathway, to disrupt the ability of CD86 to bind to CD28 and/or CTLA-4, to disrupt the ability of CD86 to activate the co-stimulatory pathway, and to disrupt the co-stimulatory pathway, in general from being activated. This necessarily includes small molecule inhibitors of CD28, CD80, CD86, CTLA-4, among other members of the co-stimulatory pathway; antibodies directed to CD28, CD80, CD86, CTLA-4, among other members of the co-stimulatory pathway; antisense molecules directed against CD28, CD80, CD86, CTLA-4, among other members of the co-stimulatory pathway; adnectins directed against CD28, CD80, CD86, CTLA-4, among other members of the co-stimulatory pathway, RNAi inhibitors (both single and double stranded) of CD28, CD80, CD86, CTLA-4, among other members of the co-stimulatory pathway, among other CTLA-4 inhibitors.

[001493] In some embodiments a CTLA-4 inhibitor binds to CTLA-4 with a K_d of about 10^{-6} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less, 10^{-10} M or less, 10^{-11} M or less, 10^{-12} M or less, *e.g.*, between 10^{-13} M and 10^{-16} M, or within any range having any two of the aforementioned values as endpoints. In some embodiments a CTLA-4 inhibitor binds to CTLA-4 with a K_d of no more than 10-fold that of ipilimumab, when compared using the same assay. In some embodiments a CTLA-4 inhibitor binds to CTLA-4 with a K_d of about the same as, or less (*e.g.*, up to 10-fold lower, or up to 100-fold lower) than that of ipilimumab, when compared using the same assay. In some embodiments, the IC_{50} values for inhibition by a CTLA-4 inhibitor of CTLA-4 binding to CD80 or CD86 is no more than 10-fold greater than that of ipilimumab-mediated inhibition of CTLA-4 binding to CD80 or CD86, respectively, when compared using the same assay. In some embodiments, the IC_{50} values for inhibition by a CTLA-4 inhibitor of CTLA-4 binding to CD80 or CD86 is about the same or less (*e.g.*, up to 10-fold lower, or up to 100-fold lower) than that of ipilimumab-mediated inhibition of CTLA-4 binding to CD80 or CD86, respectively, when compared using the same assay.

[001494] In some embodiments a CTLA-4 inhibitor is used in an amount sufficient to inhibit expression and/or decrease biological activity of CTLA-4 by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to a suitable control, *e.g.*, between 50% and 75%, 75% and 90%, or 90% and 100%. In some embodiments a CTLA-4 pathway inhibitor is used in an amount sufficient to decrease the biological activity of CTLA-4 by reducing binding of CTLA-4 to CD80, CD86, or both by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% relative to a suitable control, *e.g.*, between 50% and 75%, 75% and 90%, or 90% and 100% relative to a suitable control. A suitable control in the context of assessing or quantifying the effect of an agent of interest is typically a comparable biological system (*e.g.*, cells or a subject) that has not been exposed to or treated with the agent of interest, *e.g.*, CTLA-4 pathway inhibitor (or has been exposed to or treated with a negligible amount). In some embodiments a biological system may serve as its own control (*e.g.*, the biological system may be assessed before exposure to or treatment with the agent and compared with the state after exposure or treatment has started or finished. In some embodiments a historical control may be used.

[001495] In some embodiments, the CTLA-4 inhibitor is ipilimumab (commercially available as Yervoy from Bristol-Myers Squibb Co.), or biosimilars, antigen-binding fragments, conjugates, or variants thereof. As is known in the art, ipilimumab refers to an anti-CTLA-4 antibody, a fully human IgG 1k antibody derived from a transgenic mouse with human genes encoding heavy and light chains to generate a functional human repertoire. *is there*. Ipilimumab can also be referred to by its CAS Registry Number 477202-00-9, and in PCT Publication Number WO 01/14424, which is incorporated herein by reference in its entirety for all purposes. It is disclosed as antibody 10DI. Specifically, ipilimumab contains a light chain variable region and a heavy chain variable region (having a light chain variable region comprising SEQ ID NO:211 and having a heavy chain variable region comprising SEQ ID NO:210). A pharmaceutical composition of ipilimumab includes all pharmaceutically acceptable compositions containing ipilimumab and one or more diluents, vehicles, or excipients. An example of a pharmaceutical composition containing ipilimumab is described in International Patent Application Publication No. WO 2007/67959. Ipilimumab can be administered intravenously (IV).

[001496] In some embodiments, a CTLA-4 inhibitor comprises a heavy chain given by SEQ ID NO:208 and a light chain given by SEQ ID NO:209. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:208 and SEQ ID

NO:209, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:208 and SEQ ID NO:209, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:208 and SEQ ID NO:209, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:208 and SEQ ID NO:209, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:208 and SEQ ID NO:209, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:208 and SEQ ID NO:209, respectively.

[001497] In some embodiments, the CTLA-4 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of ipilimumab. In some embodiments, the CTLA-4 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:210, and the CTLA-4 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:211, or conservative amino acid substitutions thereof. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:210 and SEQ ID NO:211, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:210 and SEQ ID NO:211, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:210 and SEQ ID NO:211, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:210 and SEQ ID NO:211, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:210 and SEQ ID NO:211, respectively.

[001498] In some embodiments, a CTLA-4 inhibitor comprises the heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:212, SEQ ID NO:213, and SEQ ID NO:214, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:215, SEQ ID

NO:216, and SEQ ID NO:217, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on CTLA-4 as any of the aforementioned antibodies.

[001499] In some embodiments, the CTLA-4 inhibitor is a CTLA-4 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to ipilimumab. In some embodiments, the biosimilar comprises an anti-CTLA-4 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 ipilimumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. The amino acid sequences of ipilimumab are set forth in Table 24. In some embodiments, the biosimilar is an anti-CTLA-4 antibody authorized or submitted for authorization, wherein the anti-CTLA-4 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 ipilimumab. The anti-CTLA-4 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 ipilimumab. 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 ipilimumab.

TABLE 24. Amino acid sequences for ipilimumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO: 208 ipilimumab heavy chain	QVQLVESGGG VVQPGRSRLR SCAASGFTFS SYTMHWVRQA PGKGLEWVTF ISYDGNKKYY 60 ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAIYYCARTG WLGPFDYWGQ GTLVTVSSAS 120 TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL 180 YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKRVEPKS CDKTH 225

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:209 ipilimumab light chain	EIVLTQSPGT LSLSPGERAT LSCRASQSVG SSYLAWYQQK PGQAPRLLIY GAFSRATGIP 60 DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPWTFG QGTKVEIKRT VAAPSVFIIP 120 PSDEQLKSGT ASVVCLLNNF YPREAKVQWK VDNLQSGNS QESVTEQDSK DSTYLSSTL 180 TLISKADYEKH KVVACEVTHQ GLSSPVTKSF NRGEC 215
SEQ ID NO:210 ipilimumab variable heavy chain	QVQLVESGGG VVQPGRSLRL SCAASGFTFS SYTMHWVRQA PGKGLEWVTF ISYDGNKYY 60 ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAIYYCARTG WLGPFDYWGQ GTLTVVSS 118
SEQ ID NO:211 ipilimumab variable light chain	EIVLTQSPGT LSLSPGERAT LSCRASQSVG SSYLAWYQQK PGQAPRLLIY GAFSRATGIP 60 DRFSGSGSGT DFTLTISRLE PEDFAVYYCQ QYGSSPWTFG QGTKVEIK 108
SEQ ID NO:212 ipilimumab heavy chain CDR1	GFTFSSYT 8
SEQ ID NO:213 ipilimumab heavy chain CDR2	TFISYDGNK 10
SEQ ID NO:214 ipilimumab heavy chain CDR3	ARTGWLGPFD Y 11
SEQ ID NO:215 ipilimumab light chain CDR1	QSVGSSY 7
SEQ ID NO:216 ipilimumab light chain CDR2	GAF 3
SEQ ID NO:217 ipilimumab light chain CDR3	QQYGSSPWT 9

[001500] In some embodiments, the CTLA-4 inhibitor is ipilimumab or a biosimilar thereof, and the ipilimumab is administered at a dose of about 0.5 mg/kg to about 10 mg/kg. In some embodiments, the CTLA-4 inhibitor is ipilimumab or a biosimilar thereof, and the ipilimumab is administered at a dose of about 0.5 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 2.5 mg/kg, about 3 mg/kg, about 3.5 mg/kg, about 4 mg/kg, about 4.5 mg/kg, about 5 mg/kg, about 5.5 mg/kg, about 6 mg/kg, about 6.5 mg/kg, about 7 mg/kg, about 7.5 mg/kg, about 8 mg/kg, about 8.5 mg/kg, about 9 mg/kg, about 9.5 mg/kg, or about 10 mg/kg. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001501] In some embodiments, the CTLA-4 inhibitor is ipilimumab or a biosimilar thereof, and the ipilimumab is administered at a dose of about 200 mg to about 500 mg. In some embodiments, the CTLA-4 inhibitor is ipilimumab or a biosimilar thereof, and the ipilimumab is administered at a dose of about 200 mg, about 220 mg, about 240 mg, about 260 mg, about 280 mg, about 300 mg, about 320 mg, about 340 mg, about 360 mg, about 380 mg, about 400 mg,

about 420 mg, about 440 mg, about 460 mg, about 480 mg, or about 500 mg. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001502] In some embodiments, the CTLA-4 inhibitor is ipilimumab or a biosimilar thereof, and the ipilimumab is administered every 2 weeks, every 3 weeks, every 4 weeks, every 5 weeks, or every 6 weeks. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001503] In some embodiments, the ipilimumab is administered to treat unresectable or metastatic melanoma. In some embodiments, the ipilimumab is administered to treat Unresectable or Metastatic Melanoma at about mg/kg every 3 weeks for a maximum of 4 doses. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001504] In some embodiments, the ipilimumab is administered for the adjuvant treatment of melanoma. In some embodiments, the ipilimumab is administered to for the adjuvant treatment of melanoma at about 10 mg/kg every 3 weeks for 4 doses, followed by 10 mg/kg every 12 weeks for up to 3 years. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001505] In some embodiments, the ipilimumab is administered to treat advanced renal cell carcinoma. In some embodiments, the ipilimumab is administered to treat advanced renal cell carcinoma at about 1 mg/kg immediately following nivolumab 3 mg/kg on the same day, every 3 weeks for 4 doses. In some embodiments, after completing 4 doses of the combination, nivolumab can be administered as a single agent according to standard dosing regimens for

advanced renal cell carcinoma and/or renal cell carcinoma. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001506] In some embodiments, the ipilimumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer. In some embodiments, the ipilimumab is administered to treat microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer at about 1 mg/kg intravenously over 30 minutes immediately following nivolumab 3 mg/kg intravenously over 30 minutes on the same day, every 3 weeks for 4 doses. In some embodiments, after completing 4 doses of the combination, administer nivolumab as a single agent as recommended according to standard dosing regimens for microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001507] In some embodiments, the ipilimumab is administered to treat hepatocellular carcinoma. In some embodiments, the ipilimumab is administered to treat hepatocellular carcinoma at about 3 mg/kg intravenously over 30 minutes immediately following nivolumab 1 mg/kg intravenously over 30 minutes on the same day, every 3 weeks for 4 doses. In some embodiments, after completion 4 doses of the combination, administer nivolumab as a single agent according to standard dosing regimens for hepatocellular carcinoma. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001508] In some embodiments, the ipilimumab is administered to treat metastatic non-small cell lung cancer. In some embodiments, the ipilimumab is administered to treat metastatic non-small cell lung cancer at about 1 mg/kg every 6 weeks with nivolumab 3 mg/kg every 2 weeks. In

some embodiments, the ipilimumab is administered to treat metastatic non-small cell lung cancer at about 1 mg/kg every 6 weeks with nivolumab 360 mg every 3 weeks and 2 cycles of platinum-doublet chemotherapy. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001509] In some embodiments, the ipilimumab is administered to treat malignant pleural mesothelioma. In some embodiments, the ipilimumab is administered to treat malignant pleural mesothelioma at about 1 mg/kg every 6 weeks with nivolumab 360 mg every 3 weeks. In some embodiments, the ipilimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the ipilimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001510] Tremelimumab (also known as CP-675,206) is a fully human IgG2 monoclonal antibody and has the CAS number 745013-59-6. Tremelimumab is disclosed as antibody 11.2.1 in U.S. Patent No. 6,682,736 (incorporated herein by reference). The amino acid sequences of the heavy chain and light chain of tremelimumab are set forth in SEQ ID NOs:218 and 219, respectively. Tremelimumab has been investigated in clinical trials for the treatment of various tumors, including melanoma and breast cancer; in which Tremelimumab was administered intravenously either as single dose or multiple doses every 4 or 12 weeks at the dose range of 0.01 and 15 mg/kg. In the regimens provided by the present invention, tremelimumab is administered locally, particularly intradermally or subcutaneously. The effective amount of tremelimumab administered intradermally or subcutaneously is typically in the range of 5 - 200 mg/dose per person. In some embodiments, the effective amount of tremelimumab is in the range of 10 -150 mg/dose per person per dose. In some particular embodiments, the effective amount of tremelimumab is about 10, 25, 37.5, 40, 50, 75, 100, 125, 150, 175, or 200 mg/dose per person.

[001511] In some embodiments, a CTLA-4 inhibitor comprises a heavy chain given by SEQ ID NO:218 and a light chain given by SEQ ID NO:219. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:218 and SEQ ID

NO:219, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:218 and SEQ ID NO:219, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:218 and SEQ ID NO:219, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:218 and SEQ ID NO:219, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:218 and SEQ ID NO:219, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:218 and SEQ ID NO:219, respectively.

[001512] In some embodiments, the CTLA-4 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of tremelimumab. In some embodiments, the CTLA-4 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:220, and the CTLA-4 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:221, or conservative amino acid substitutions thereof. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:220 and SEQ ID NO:221, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:220 and SEQ ID NO:221, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:220 and SEQ ID NO:221, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:220 and SEQ ID NO:221, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:220 and SEQ ID NO:221, respectively.

[001513] In some embodiments, a CTLA-4 inhibitor comprises the heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:222, SEQ ID NO:223, and SEQ ID NO:224, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:225, SEQ ID

NO:226, and SEQ ID NO:227, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on CTLA-4 as any of the aforementioned antibodies.

[001514] In some embodiments, the CTLA-4 inhibitor is an anti-CTLA-4 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to tremelimumab. In some embodiments, the biosimilar comprises an anti-CTLA-4 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 tremelimumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. The amino acid sequences of tremelimumab are set forth in Table 25. In some embodiments, the biosimilar is an anti-CTLA-4 antibody authorized or submitted for authorization, wherein the anti-CTLA-4 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 tremelimumab. The anti-CTLA-4 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 tremelimumab. 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 tremelimumab.

TABLE 25. Amino acid sequences for tremelimumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:218 tremelimumab heavy chain	QVQLVESGGG VVQPGRSRLR SCAASGFTFS SYGMHWVRQA PGKGLEWVAV IWYDGSNKYY 60 ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCARDP RGATLYYYYY GMDVWGQGT 120 VTVSSASTKG PSVFPLAPCS RSTSESTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA 180 VLQSSGLYSL SSVVTVPSSN FGTQYITCNV DHKPSNTKVD KTVERKCCVE CPPCPAPPVA 240

Identifier	Sequence (One-Letter Amino Acid Symbols)
	GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVQFN WYVDGVEVHN AKTKPREEQF 300 NSTFRVSVL TVVHQDWLNG KEYKCKVSNK GLPAPIEKTI SKTKGQPREP QVYTLPPSRE 360 EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTTP MLDSGGSFFL YSKLTVDKSR 420 WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K 451
SEQ ID NO: 219 tremelimumab light chain	DIQMTQSPSS LSASVGDRVT ITCRASQIN SYLDWYQQKPKAPKLLIYA ASSLSQSGVPS 60 RFGSGSGTD FTLTISSLQP EDFATYYCQQ YYSTPFTFGP GTKVEIKRTV AAPSVFIFPP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSLSTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEK 214
SEQ ID NO: 220 tremelimumab variable heavy chain	GVVQPGRLR LSCAASGFTF SSYGMHWVRQ APGKGLEWVA VIWYDGSNKY YADSVKGRFT 60 ISRDNKNTL YLQMNSLRAE DTAVYYCARD PRGATLYYYY YGMDVWGQGT TVTVSSASTK 120 GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVH 167
SEQ ID NO: 221 tremelimumab variable light chain	PSSLSASVGD RVTITCRASQ SINSYLDWYQ QKPGKAPKLL IYAASSLSQSG VPSRFGSGS 60 GTDFTLTIS LQPEDFATYY CQYYSTPFT FPGGTKVEIK RTVAAPSVEI FPPSDEQLKS 120 GTASVVCLLN NFYPREAKV 139
SEQ ID NO: 222 tremelimumab heavy chain CDR1	GFTFSSYGMH 10
SEQ ID NO: 223 tremelimumab heavy chain CDR2	VIWYDGSNKY YADSV 15
SEQ ID NO: 224 tremelimumab heavy chain CDR3	DPRGATLYYYY YGMDV 16
SEQ ID NO: 225 tremelimumab light chain CDR1	RASQSINSYL D 11
SEQ ID NO: 226 tremelimumab light chain CDR2	AASSLSQ 7
SEQ ID NO: 227 tremelimumab light chain CDR3	QYYSTPFT 9

[0006] In some embodiments, the CTLA-4 inhibitor is tremelimumab or a biosimilar thereof, and the tremelimumab is administered at a dose of about 0.5 mg/kg to about 10 mg/kg. In some embodiments, the CTLA-4 inhibitor is tremelimumab or a biosimilar thereof, and the tremelimumab is administered at a dose of about 0.5 mg/kg, about 1 mg/kg, about 1.5 mg/kg, about 2 mg/kg, about 2.5 mg/kg, about 3 mg/kg, about 3.5 mg/kg, about 4 mg/kg, about 4.5 mg/kg, about 5 mg/kg, about 5.5 mg/kg, about 6 mg/kg, about 6.5 mg/kg, about 7 mg/kg, about 7.5 mg/kg, about 8 mg/kg, about 8.5 mg/kg, about 9 mg/kg, about 9.5 mg/kg, or about 10 mg/kg. In some embodiments, the tremelimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the tremelimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[0007] In some embodiments, the CTLA-4 inhibitor is tremelimumab or a biosimilar thereof, and the tremelimumab is administered at a dose of about 200 mg to about 500 mg. In some embodiments, the CTLA-4 inhibitor is tremelimumab or a biosimilar thereof, and the tremelimumab is administered at a dose of about 200 mg, about 220 mg, about 240 mg, about 260 mg, about 280 mg, about 300 mg, about

320 mg, about 340 mg, about 360 mg, about 380 mg, about 400 mg, about 420 mg, about 440 mg, about 460 mg, about 480 mg, or about 500 mg. In some embodiments, the tremelimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the tremelimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[0008] In some embodiments, the CTLA-4 inhibitor is tremelimumab or a biosimilar thereof, and the tremelimumab is administered every 2 weeks, every 3 weeks, every 4 weeks, every 5 weeks, or every 6 weeks. In some embodiments, the tremelimumab administration is begun 1, 2, 3, 4, or 5 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient). In some embodiments, the tremelimumab administration is begun 1, 2, or 3 weeks pre-resection (*i.e.*, prior to obtaining the tumor sample from the subject or patient).

[001515] In some embodiments, the CTLA-4 inhibitor is zalifrelimab from Agenus, or biosimilars, antigen-binding fragments, conjugates, or variants thereof. Zalifrelimab is a fully human monoclonal antibody. Zalifrelimab is assigned Chemical Abstracts Service (CAS) registry number 2148321-69-9 and is also known as also known as AGEN1884. The preparation and properties of zalifrelimab are described in U.S. Patent No. 10,144,779 and US Patent Application Publication No. US2020/0024350 A1, the disclosures of which are incorporated by reference herein.

[001516] In some embodiments, a CTLA-4 inhibitor comprises a heavy chain given by SEQ ID NO:228 and a light chain given by SEQ ID NO:229. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains having the sequences shown in SEQ ID NO:228 and SEQ ID NO:229, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:228 and SEQ ID NO:229, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:228 and SEQ ID NO:229, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:228 and SEQ ID NO:229, respectively. In some embodiments, a CTLA-4 inhibitor comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:228 and SEQ ID NO:229, respectively. In some embodiments, a CTLA-4 inhibitor

comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:228 and SEQ ID NO:229, respectively.

[001517] In some embodiments, the CTLA-4 inhibitor comprises the heavy and light chain CDRs or variable regions (VRs) of zalifrelimab. In some embodiments, the CTLA-4 inhibitor heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:230, and the CTLA-4 inhibitor light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:231, or conservative amino acid substitutions thereof. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:230 and SEQ ID NO:231, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:230 and SEQ ID NO:231, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:230 and SEQ ID NO:231, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:230 and SEQ ID NO:231, respectively. In some embodiments, a CTLA-4 inhibitor comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:230 and SEQ ID NO:231, respectively.

[001518] In some embodiments, a CTLA-4 inhibitor comprises the heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:231, SEQ ID NO:233, and SEQ ID NO:234, respectively, or conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:235, SEQ ID NO:236, and SEQ ID NO:237, respectively, or conservative amino acid substitutions thereof. In some embodiments, the antibody competes for binding with, and/or binds to the same epitope on CTLA-4 as any of the aforementioned antibodies.

[001519] In some embodiments, the CTLA-4 inhibitor is a CTLA-4 biosimilar monoclonal antibody approved by drug regulatory authorities with reference to zalifrelimab. In some embodiments, the biosimilar comprises an anti-CTLA-4 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 zalifrelimab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. The amino acid sequences of zalifrelimab are set forth in Table 26. In some embodiments, the biosimilar is an anti-CTLA-4 antibody authorized or submitted for authorization, wherein the anti-CTLA-4 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 zalifrelimab. The anti-CTLA-4 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 zalifrelimab. 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 zalifrelimab.

TABLE 26. Amino acid sequences for zalifrelimab.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:228 zalifrelimab heavy chain	EVQLVESGGG LVKPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSS ISSSSSYIYY 60 ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARVG LMGPFDIWGQ GTMVTVSSAS 120 TKGPSVFPLA PSSKSTSGGT AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL 180 YSLSSVVTVP SSSLGTQTYI CNVNHKPSNT KVDKRVEPKS CDKTHTCPPC PAPELLGGPS 240 VFLFPPKPKD TLMISRTPEV TCVVVDVSH E DPEVKFNWYV DGVEVHNAKT KPREEQYNST 300 YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA KGQPREPQVY TLPPSREEMT 360 KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTTPVLD SDGSFFLYSK LTVDKSRWQQ 420 GNVFSCVMH EALHNHYTQK SLSLSPGK 448
SEQ ID NO:229 zalifrelimab light chain	EIVLTQSPGT LSLSPGERAT LSCRASQSVS RYLGWYQQKP GQAPRLLIYG ASTRATGIPD 60 RFGSGSGTD FTLTITRLEP EDFAVYYCQQ YGSSPWTFGQ GTKVEIKRTV AAPSVFIFPP 120 SDEQLKSGTA SVVCLLNIFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYLSSTLT 180 LSKADYEKHK VYACEVTHQG LSSPVTGSFN RGEC 214
SEQ ID NO:230 zalifrelimab variable heavy chain	EVQLVESGGG LVKPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSS ISSSSSYIYY 60 ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARVG LMGPFDIWGQ GTMVTVSS 118
SEQ ID NO:231 zalifrelimab variable light chain	EIVLTQSPGT LSLSPGERAT LSCRASQSVS RYLGWYQQKP GQAPRLLIYG ASTRATGIPD 60 RFGSGSGTD FTLTITRLEP EDFAVYYCQQ YGSSPWTFGQ GTKVEIK 107
SEQ ID NO:232 zalifrelimab heavy chain CDR1	GFTSSYS 8
SEQ ID NO:233 zalifrelimab heavy chain CDR2	ISSSSSYI 8
SEQ ID NO:234	ARVGLMGPFDI 11

Identifier	Sequence (One-Letter Amino Acid Symbols)
zalifrelimab heavy chain CDR3	
SEQ ID NO:235 zalifrelimab light chain CDR1	QSVSR Y 6
SEQ ID NO:236 zalifrelimab light chain CDR2	GAS 3
SEQ ID NO:237 zalifrelimab light chain CDR3	QQYGSSPWT 9

[001520] Examples of additional anti-CTLA-4 antibodies includes, but are not limited to: AGEN1181, BMS-986218, BCD-145, ONC-392, CS1002, REGN4659, and ADG116, which are known to one of ordinary skill in the art.

[001521] In some embodiments, the anti-CTLA-4 antibody is an anti-CTLA-4 antibody disclosed in any of the following patent publications: US 2019/0048096 A1; US 2020/0223907; US 2019/0201334; US 2019/0201334; US 2005/0201994; EP 1212422 B1; WO 2018/204760; WO 2018/204760; WO 2001/014424; WO 2004/035607; WO 2003/086459; WO 2012/120125; WO 2000/037504; WO 2009/100140; WO 2006/09649; WO2005092380; WO 2007/123737; WO 2006/029219; WO 2010/0979597; WO 2006/12168; and WO1997020574, each of which is incorporated herein by reference. Additional CTLA-4 antibodies are described in U.S. Pat. Nos. 5,811,097, 5,855,887, 6,051,227, and 6,984,720; in PCT Publication Nos. WO 01/14424 and WO 00/37504; and in U.S. Publication Nos. 2002/0039581 and 2002/086014; and/or U.S. Patent Nos. 5,977,318, 6,682,736, 7,109,003, and 7,132,281, each of which is incorporated herein by reference. In some embodiments, the anti-CTLA-4 antibody is, for example, those disclosed in: WO 98/42752; U.S. Pat. Nos. 6,682,736 and 6,207,156; Hurwitz, *et al.*, *Proc. Natl. Acad. Sci. USA*, **1998**, 95, 10067-10071 (1998); Camacho, *et al.*, *J. Clin. Oncol.*, **2004**, 22, 145 (Abstract No. 2505 (2004) (antibody CP-675206); or Mokyr, *et al.*, *Cancer Res.*, **1998**, 58, 5301-5304 (1998), each of which is incorporated herein by reference.

[001522] In some embodiments, the CTLA-4 inhibitor is a CTLA-4 ligand as disclosed in WO 1996/040915 (incorporated herein by reference).

[001523] In some embodiments, the CTLA-4 inhibitor is a nucleic acid inhibitor of CTLA-4 expression. For example, anti-CTLA-4 RNAi molecules may take the form of the molecules described in PCT Publication Nos. WO 1999/032619 and WO 2001/029058; U.S. Publication

Nos. 2003/0051263, 2003/0055020, 2003/0056235, 2004/265839, 2005/0100913, 2006/0024798, 2008/0050342, 2008/0081373, 2008/0248576, and 2008/055443; and/or U.S. Pat. Nos. 6,506,559, 7,282,564, 7,538,095, and 7,560,438 (incorporated herein by reference). In some instances, the anti-CTLA-4 RNAi molecules take the form of double stranded RNAi molecules described in European Patent No. EP 1309726 (incorporated herein by reference). In some instances, the anti-CTLA-4 RNAi molecules take the form of double stranded RNAi molecules described in U.S. Pat. Nos. 7,056,704 and 7,078,196 (incorporated herein by reference). In some embodiments, the CTLA-4 inhibitor is an aptamer described in International Patent Application Publication No. WO 2004/081021 (incorporated herein by reference).

[001524] In other embodiments, the anti-CTLA-4 RNAi molecules of the present invention are RNA molecules described in U.S. Patent Nos. 5,898,031, 6,107,094, 7,432,249, and 7,432,250, and European Application No. EP 0928290 (incorporated herein by reference).

3. Lymphodepletion Preconditioning of Patients

[001525] In some embodiments, 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 some embodiments, 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 some embodiments, the population of TILs is for administration by infusion. In some embodiments, 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 some embodiments, 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.

[001526] 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.

[001527] 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.

[001528] In some embodiments, the fludarabine is administered at a concentration of 0.5 µg/mL to 10 µg/mL fludarabine. In some embodiments, the fludarabine is administered at a concentration of 1 µg/mL fludarabine. In some embodiments, the fludarabine treatment is administered for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days or more. In some embodiments, the fludarabine is administered at a dosage of 10 mg/kg/day, 15 mg/kg/day, 20 mg/kg/day, 25 mg/kg/day, 30 mg/kg/day, 35 mg/kg/day, 40 mg/kg/day, or 45 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 2–7 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4–5 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4–5 days at 25 mg/kg/day.

[001529] In some embodiments, the mafosfamide, the active form of cyclophosphamide, is obtained at a concentration of 0.5 µg/mL to 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.e.*, i.v.) In some embodiments, the cyclophosphamide treatment is administered for 2–7 days at 35 mg/kg/day. In some embodiments, the cyclophosphamide treatment is administered for 4–5 days at 250 mg/m²/day i.v. In some embodiments, the cyclophosphamide treatment is administered for 4 days at 250 mg/m²/day i.v.

[001530] 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.

[001531] In some embodiments, 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.

[001532] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m²/day for two days and administration of fludarabine at a dose of 25 mg/m²/day for five days, wherein cyclophosphamide and fludarabine are both administered on the first two days, and wherein the lymphodepletion is performed in five days in total.

[001533] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 50 mg/m²/day for two days and administration of fludarabine at a dose of about 25 mg/m²/day for five days, wherein cyclophosphamide and fludarabine are both administered on the first two days, and wherein the lymphodepletion is performed in five days in total.

[001534] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 50 mg/m²/day for two days and administration of fludarabine at a dose of about 20 mg/m²/day for five days, wherein cyclophosphamide and fludarabine are both administered on the first two days, and wherein the lymphodepletion is performed in five days in total.

[001535] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 40 mg/m²/day for two days and administration of fludarabine at a dose of about 20 mg/m²/day for five days, wherein cyclophosphamide and fludarabine are both administered on the first two days, and wherein the lymphodepletion is performed in five days in total.

[001536] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 40 mg/m²/day for two days and administration of

fludarabine at a dose of about 15 mg/m²/day for five days, wherein cyclophosphamide and fludarabine are both administered on the first two days, and wherein the lymphodepletion is performed in five days in total.

[001537] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m²/day and fludarabine at a dose of 25 mg/m²/day for two days followed by administration of fludarabine at a dose of 25 mg/m²/day for three days.

[001538] In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, mesna is administered at 15 mg/kg. In some embodiments where mesna is infused, and if infused continuously, mesna can be infused over approximately 2 hours with cyclophosphamide (on Days -5 and/or -4), then at a rate of 3 mg/kg/hour for the remaining 22 hours over the 24 hours starting concomitantly with each cyclophosphamide dose.

[001539] In some embodiments, the lymphodepletion comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

[001540] In some embodiments, the lymphodepletion comprises the step of treating the patient with an IL-2 regimen starting on the same day as administration of the third population of TILs to the patient.

[001541] In some embodiments, the lymphodeplete comprises 5 days of preconditioning treatment. In some embodiments, the days are indicated as days -5 through -1, or Day 0 through Day 4. In some embodiments, the regimen comprises cyclophosphamide on days -5 and -4 (*i.e.*, days 0 and 1). In some embodiments, the regimen comprises intravenous cyclophosphamide on days -5 and -4 (*i.e.*, days 0 and 1). In some embodiments, the regimen comprises 60 mg/kg intravenous cyclophosphamide on days -5 and -4 (*i.e.*, days 0 and 1). In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the regimen further comprises fludarabine. In some embodiments, the regimen further comprises intravenous fludarabine. In some embodiments, the regimen further comprises 25 mg/m² intravenous fludarabine. In some embodiments, the regimen further comprises 25 mg/m² intravenous fludarabine on days -5 and -1 (*i.e.*, days 0 through 4). In some embodiments, the regimen further comprises 25 mg/m² intravenous fludarabine on days -5 and -1 (*i.e.*, days 0 through 4).

[001542] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m²/day and fludarabine at a dose of 25 mg/m²/day for two days followed by administration of fludarabine at a dose of 25 mg/m²/day for five days.

[001543] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of 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.

[001544] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of 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 three days

[001545] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m²/day and fludarabine at a dose of 25 mg/m²/day for two days followed by administration of fludarabine at a dose of 25 mg/m²/day for three days.

[001546] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m²/day and fludarabine at a dose of 25 mg/m²/day for two days followed by administration of fludarabine at a dose of 25 mg/m²/day for one day.

[001547] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of 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 three days.

[001548] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m²/day and fludarabine at a dose of 25 mg/m²/day for two days followed by administration of fludarabine at a dose of 25 mg/m²/day for three days.

[001549] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 27.

TABLE 27. Exemplary lymphodepletion and treatment regimen.

Day	-5	-4	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 60 mg/kg	X	X								
Mesna (as needed)	X	X								
Fludarabine 25 mg/m ² /day	X	X	X	X	X					
TIL infusion						X				

[001550] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 28.

TABLE 28. Exemplary lymphodepletion and treatment regimen.

Day	-4	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 60 mg/kg	X	X							
Mesna (as needed)	X	X							
Fludarabine 25 mg/m ² /day	X	X	X	X					
TIL infusion					X				

[001551] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 29.

TABLE 29. Exemplary lymphodepletion and treatment regimen.

Day	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 60 mg/kg	X	X						
Mesna (as needed)	X	X						
Fludarabine 25 mg/m ² /day	X	X	X					
TIL infusion				X				

[001552] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 30.

TABLE 30. Exemplary lymphodepletion and treatment regimen.

Day	-5	-4	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 60 mg/kg	X	X								
Mesna (as needed)	X	X								
Fludarabine 25 mg/m ² /day			X	X	X					

Day	-5	-4	-3	-2	-1	0	1	2	3	4
TIL infusion						X				

[001553] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 31.

TABLE 31. Exemplary lymphodepletion and treatment regimen.

Day	-5	-4	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 300 mg/kg	X	X								
Mesna (as needed)	X	X								
Fludarabine 30 mg/m ² /day	X	X	X	X	X					
TIL infusion						X				

[001554] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 32.

TABLE 32. Exemplary lymphodepletion and treatment regimen.

Day	-4	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 300 mg/kg	X	X							
Mesna (as needed)	X	X							
Fludarabine 30 mg/m ² /day	X	X	X	X					
TIL infusion					X				

[001555] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 33.

TABLE 33. Exemplary lymphodepletion and treatment regimen.

Day	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 300 mg/kg	X	X						
Mesna (as needed)	X	X						
Fludarabine 30 mg/m ² /day	X	X	X					
TIL infusion				X				

[001556] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 34.

TABLE 34. Exemplary lymphodepletion and treatment regimen.

Day	-5	-4	-3	-2	-1	0	1	2	3	4
Cyclophosphamide 300 mg/kg	X	X								
Mesna (as needed)	X	X								
Fludarabine 30 mg/m ² /day			X	X	X					
TIL infusion						X				

[0009] In some embodiments, the TIL infusion used with the foregoing embodiments of myeloablative lymphodepletion regimens may be any TIL composition described herein, as well as the addition of IL-2 regimens and administration of co-therapies (such as PD-1 and PD-L1 inhibitors) as described herein.

4. IL-2 Regimens

[001557] In some embodiments, 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. In some embodiments, IL-2 is administered in 1, 2, 3, 4, 5, or 6 doses. In some embodiments, IL-2 is administered at a maximum dosage of up to 6 doses.

[001558] In some embodiments, 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 some embodiments, a decrescendo IL-2 regimen comprises 18×10^6 IU/m² aldesleukin, or a biosimilar or variant thereof, 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 hours, 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 some embodiments, 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.

[0010] In some embodiments, the IL-2 regimen comprises a low-dose IL-2 regimen. Any low-dose IL-2 regimen known in the art may be used, including the low-dose IL-2 regimens described in Dominguez-Villar and Hafler, *Nat. Immunology* **2000**, 19, 665-673; Hartmann, *et al.*, *Lancet Diabetes Endocrinol.* **2013**, 1, 295-305; and Rosenzwaig, *et al.*, *Ann. Rheum. Dis.* **2019**, 78, 209-217, the disclosures of which are incorporated herein by reference. In some embodiments, a low-dose IL-2 regimen comprises 18×10^6 IU per m² of aldesleukin, or a biosimilar or variant thereof, per 24 hours, administered as a continuous infusion for 5 days, followed by 2-6 days without IL-2 therapy, optionally followed by an additional 5 days of intravenous aldesleukin or a biosimilar or variant thereof, as a continuous infusion of 18×10^6 IU per m² per 24 hours, optionally followed by 3 weeks without IL-2 therapy, after which additional cycles may be administered.

[001559] In some embodiments, IL-2 is administered at a maximum dosage of up to 6 doses. In some embodiments, the high-dose IL-2 regimen is adapted for pediatric use. In some embodiments, a dose of 600,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used. In some embodiments, a dose of 500,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used. In some embodiments, a dose of 400,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used. In some embodiments, a dose of 500,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used. In some embodiments, a dose of 300,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used. In some embodiments, a dose of 200,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used. In some embodiments, a dose of 100,000 international units (IU)/kg of aldesleukin every 8-12 hours for up to a maximum of 6 doses is used.

[001560] In some embodiments, 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. In some embodiments, the IL-2 regimen comprises administration of bempegaldesleukin, or a fragment, variant, or biosimilar thereof, every 1, 2, 4, 6, 7, 14 or 21 days at a dose of 0.10 mg/day to 50 mg/day.

[001561] In some embodiments, the IL-2 regimen comprises administration of THOR-707, or a fragment, variant, or biosimilar thereof, every 1, 2, 4, 6, 7, 14 or 21 days at a dose of 0.10 mg/day to 50 mg/day.

[001562] In some embodiments, the IL-2 regimen comprises administration of nemvaleukin alfa, or a fragment, variant, or biosimilar thereof, following administration of TIL. In certain embodiments, the patient the nemvaleukin is administered every 1, 2, 4, 6, 7, 14 or 21 days at a dose of 0.10 mg/day to 50 mg/day.

[001563] In some embodiments, the IL-2 regimen comprises administration of an IL-2 fragment engrafted onto an antibody backbone. In some embodiments, the IL-2 regimen comprises administration of an antibody-cytokine engrafted protein that binds the IL-2 low affinity receptor. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain variable region (V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L , wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain variable region (V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L , wherein the IL-2 molecule is a mutein, and wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the IL-2 regimen comprises administration of an antibody comprising a heavy chain selected from the group consisting of SEQ ID NO:29 and SEQ ID NO:38 and a light chain selected from the group consisting of SEQ ID NO:37 and SEQ ID NO:39, or a fragment, variant, or biosimilar thereof, every 1, 2, 4, 6, 7, 14 or 21 days at a dose of 0.10 mg/day to 50 mg/day.

[001564] In some embodiments, the antibody cytokine engrafted protein described herein has a longer serum half-life than a wild-type IL-2 molecule such as, but not limited to, aldesleukin (Proleukin®) or a comparable molecule.

[001565] In some embodiments, the TIL infusion used with the foregoing embodiments of myeloablative lymphodepletion regimens may be any TIL composition described herein and may also include infusions of MILs and PBLs in place of the TIL infusion, as well as the addition of IL-2 regimens and administration of co-therapies (such as PD-1 and/or PD-L1 inhibitors and/or CTLA-4 inhibitors) as described herein.

V. EXAMPLES

[001566] 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

[001567] 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 solid tumors. This media can be used for preparation of any of the TILs described in the present application and other examples.

[001568] Preparation of CM1. Removed the following reagents from cold storage and warm them in a 37°C water bath: (RPMI1640, Human AB serum, 200 mM L-glutamine). Prepared CM1 medium according to Table 35 below by adding each of the ingredients into the top section of a 0.2 µm filter unit appropriate to the volume to be filtered. Store at 4°C.

TABLE 35. Preparation of CM1

Ingredient	Final concentration	Final Volume 500 mL	Final Volume IL
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 µM	0.5 mL	1 mL
50mg/mL gentamicin sulfate	50 µg/mL	0.5 mL	1 mL

[001569] On the day of use, prewarmed required amount of CM1 in 37°C water bath and add 6000 IU/mL IL-2.

[001570] Additional supplementation may be performed as needed according to Table 36.

TABLE 36. 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 µg/mL streptomycin	1:100	100 U/mL penicillin 100 µg/mL streptomycin
Amphotericin B	250 µg/mL	1:100	2.5 µg/mL

Preparation of CM2

[001571] Removed prepared CM1 from refrigerator or prepare fresh CM1. 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

[001572] Prepared CM3 on the day it was required for use. CM3 was the same as AIM-V® medium, supplemented with 3000 IU/mL IL-2 on the day of use. Prepared an amount of CM3 sufficient to experimental needs by adding IL-2 stock solution directly to the bottle or bag of AIM-V. Mixed well by gentle shaking. Label bottle with “3000 IU/mL IL-2” immediately after adding to the AIM-V. If there was excess CM3, stored it in bottles at 4°C labeled with the media name, the initials of the preparer, the date the media was prepared, and its expiration date (7 days after preparation). Discarded media supplemented with IL-2 after 7 days storage at 4°C.

Preparation of CM4

[001573] CM4 was the same as CM3, with the additional supplement of 2mM GlutaMAX™ (final concentration). For every 1L of CM3, add 10 mL of 200 mM GlutaMAX™. Prepare 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 IL/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 more than 7-days storage at 4°C.

EXAMPLE 2: USE OF IL-2, IL-15, AND IL-21 CYTOKINE COCKTAIL

[001574] 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 herein.

[001575] Using the processes described herein, TILs can be grown from 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 V β repertoire. IL-15 and IL-21 are described elsewhere herein and in Santegoets, *et al.*, *J. Transl. Med.*, **2013**, *11*, 37.

[001576] The results can show that enhanced TIL expansion (>20%), in both CD4⁺ and CD8⁺ cells in the IL-2, IL-15, and IL-21 treated conditions can observed relative to the IL-2 only conditions. There was a skewing towards a predominantly CD8⁺ population with a skewed TCR 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: QUALIFYING INDIVIDUAL LOTS OF GAMMA-IRRADIATED PERIPHERAL MONONUCLEAR CELLS

[001577] This Example describes an abbreviated procedure for qualifying individual lots of gamma-irradiated peripheral mononuclear cells (PBMCs, also known as mononuclear cells or MNCs) for use as allogeneic feeder cells in the exemplary methods described herein.

[001578] Each irradiated MNC feeder lot was prepared from an individual donor. Each lot or donor was screened individually for its ability to expand TIL in the REP in the presence of purified anti-CD3 (clone OKT3) antibody and interleukin-2 (IL-2). In addition, each lot of feeder cells was tested without the addition of TIL to verify that the received dose of gamma radiation was sufficient to render them replication incompetent.

[001579] Gamma-irradiated, growth-arrested MNC feeder cells are required for REP of TILs. Membrane receptors on the feeder MNCs bind to anti-CD3 (clone OKT3) antibody and crosslink to TILs in the REP flask, stimulating the TIL to expand. Feeder lots were prepared from the leukapheresis of whole blood taken from individual donors. The leukapheresis product was subjected to centrifugation over Ficoll-Hypaque, washed, irradiated, and cryopreserved under GMP conditions.

[001580] It is important that patients who received TIL therapy not be infused with viable feeder cells as this can result in graft-versus-host disease (GVHD). Feeder cells are therefore growth-arrested by dosing the cells with gamma-irradiation, resulting in double strand DNA breaks and the loss of cell viability of the MNC cells upon re-culture.

[001581] Feeder lots were evaluated on two criteria: (1) their ability to expand TILs in co-culture >100-fold and (2) their replication incompetency.

[001582] Feeder lots were tested in mini-REP format utilizing two primary pre-REP TIL lines grown in upright T25 tissue culture flasks. Feeder lots were tested against two distinct TIL lines, as each TIL line is unique in its ability to proliferate in response to activation in a REP. As a control, a lot of irradiated MNC feeder cells which has historically been shown to meet the criteria above was run alongside the test lots.

[001583] To ensure that all lots tested in a single experiment receive equivalent testing, sufficient stocks of the same pre-REP TIL lines were available to test all conditions and all feeder lots.

[001584] For each lot of feeder cells tested, there was a total of six T25 flasks: Pre-REP TIL line #1 (2 flasks); Pre-REP TIL line #2 (2 flasks); and feeder control (2 flasks). Flasks containing TIL lines #1 and #2 evaluated the ability of the feeder lot to expand TIL. The feeder control flasks evaluated the replication incompetence of the feeder lot.

A. Experimental Protocol

[001585] Day -2/3, Thaw of TIL lines. Prepare CM2 medium and warm CM2 in 37°C water bath. Prepare 40 mL of CM2 supplemented with 3000 IU/mL IL-2. Keep warm until use. Place 20 mL of pre-warmed CM2 without IL-2 into each of two 50 mL conical tubes labeled with names of the TIL lines used. Removed the two designated pre-REP TIL lines from LN2

storage and transferred the vials to the tissue culture room. Thawed vials by placing them inside a sealed zipper storage bag in a 37°C water bath until a small amount of ice remains.

[001586] Using a sterile transfer pipet, the contents of each vial were immediately transferred into the 20 mL of CM2 in the prepared, labeled 50 mL conical tube. QS to 40 mL using CM2 without IL-2 to wash cells and centrifuged at $400 \times \text{CF}$ for 5 minutes. Aspirated the supernatant and resuspend in 5 mL warm CM2 supplemented with 3000 IU/mL IL-2.

[001587] A small aliquot (20 μL) was removed in duplicate for cell counting using an automated cell counter. The counts were recorded. While counting, the 50 mL conical tube with TIL cells was placed into a humidified 37°C, 5% CO_2 incubator, with the cap loosened to allow for gas exchange. The cell concentration was determined, and the TILs were diluted to 1×10^6 cells/mL in CM2 supplemented with IL-2 at 3000 IU/mL.

[001588] Cultured in 2 mL/well of a 24-well tissue culture plate in as many wells as needed in a humidified 37°C incubator until Day 0 of the mini-REP. The different TIL lines were cultured in separate 24-well tissue culture plates to avoid confusion and potential cross-contamination.

[001589] Day 0, initiate Mini-REP. Prepared enough CM2 medium for the number of feeder lots to be tested. (e.g., for testing 4 feeder lots at one time, prepared 800 mL of CM2 medium). Aliquoted a portion of the CM2 prepared above and supplemented it with 3000 IU/mL IL-2 for the culturing of the cells. (e.g., for testing 4 feeder lots at one time, prepare 500 mL of CM2 medium with 3000 IU/mL IL-2).

[001590] Working with each TIL line separately to prevent cross-contamination, the 24-well plate with TIL culture was removed from the incubator and transferred to the BSC.

[001591] Using a sterile transfer pipet or 100-1000 μL pipettor and tip, about 1 mL of medium was removed from each well of TILs to be used and placed in an unused well of the 24-well tissue culture plate.

[001592] Using a fresh sterile transfer pipet or 100-1000 μL pipettor and tip, the remaining medium was mixed with TILs in wells to resuspend the cells and then transferred the cell suspension to a 50 mL conical tube labeled with the TIL lot name and recorded the volume.

[001593] Washed the wells with the reserved media and transferred that volume to the same 50 mL conical tube. Spun the cells at $400 \times \text{CF}$ to collect the cell pellet. Aspirated off the media supernatant and resuspend the cell pellet in 2-5 mL of CM2 medium containing 3000 IU/mL IL-2, volume to be used based on the number of wells harvested and the size of the pellet – volume should be sufficient to ensure a concentration of $>1.3 \times 10^6$ cells/mL.

[001594] Using a serological pipet, the cell suspension was mixed thoroughly and the volume was recorded. Removed 200 μL for a cell count using an automated cell counter. While counting, placed the 50 mL conical tube with TIL cells into a humidified, 5% CO_2 , 37°C incubator, with the cap loosened to allow gas exchange. Recorded the counts.

[001595] Removed the 50 mL conical tube containing the TIL cells from the incubator and resuspend them cells at a concentration of 1.3×10^6 cells/mL in warm CM2 supplemented with 3000 IU/mL IL-2. Returned the 50 mL conical tube to the incubator with a loosened cap.

[001596] The steps above were repeated for the second TIL line.

[001597] Just prior to plating the TIL into the T25 flasks for the experiment, TIL were diluted 1:10 for a final concentration of 1.3×10^5 cells/mL as per below.

[001598] Prepare MACS GMP CD3 pure (OKT3) working solution. Took out stock solution of OKT3 (1 mg/mL) from 4°C refrigerator and placed in BSC. A final concentration of 30 ng/mL OKT3 was used in the media of the mini-REP.

[001599] 600 ng of OKT3 were needed for 20 mL in each T25 flask of the experiment; this was the equivalent of 60 μL of a 10 $\mu\text{g/mL}$ solution for each 20 mL, or 360 μL for all 6 flasks tested for each feeder lot.

[001600] For each feeder lot tested, made 400 μL of a 1:100 dilution of 1 mg/mL OKT3 for a working concentration of 10 $\mu\text{g/mL}$ (*e.g.*, for testing 4 feeder lots at one time, make 1600 μL of a 1:100 dilution of 1 mg/mL OKT3: 16 μL of 1 mg/mL OKT3 + 1.584 mL of CM2 medium with 3000 IU/mL IL-2.)

[001601] Prepare T25 flasks. Labeled each flask and filled flask with the CM2 medium prior to preparing the feeder cells. Placed flasks into 37°C humidified 5% CO_2 incubator to keep media warm while waiting to add the remaining components. Once feeder cells were prepared, the components will be added to the CM2 in each flask.

[001602] Further information is provided in Table 37.

TABLE 37. Solution information.

Component	Volume in co-culture flasks	Volume in control (feeder only) flasks
CM2 + 3000 IU/mL IL-2	18 mL	19 mL
MNC: 1.3×10^7 /mL in CM2 + 3000 IU IL-2 (final concentration 1.3×10^7 /flask)	1 mL	1 mL
OKT3: 10 μ L/mL in CM2 = 3000 IU IL-2	60 μ L	60 μ L
TIL: 1.3×10^5 /mL in CM2 with 3000 IU of IL-2 (final concentration 1.3×10^5 /flask)	1 mL	0

[001603] Prepare Feeder Cells. A minimum of 78×10^6 feeder cells were needed per lot tested for this protocol. Each 1 mL vial frozen by SDBB had 100×10^6 viable cells upon freezing. Assuming a 50% recovery upon thaw from liquid N₂ storage, it was recommended to thaw at least two 1 mL vials of feeder cells per lot giving an estimated 100×10^6 viable cells for each REP. Alternately, if supplied in 1.8 mL vials, only one vial provided enough feeder cells.

[001604] Before thawing feeder cells, approximately 50 mL of CM2 without IL-2 was pre-warmed for each feeder lot to be tested. The designated feeder lot vials were removed from LN₂ storage, placed in zipper storage bag, and placed on ice. Vials were thawed inside closed zipper storage bag by immersing in a 37°C water bath. Vials were removed from zipper bag, sprayed or wiped with 70% EtOH, and transferred to a BSC.

[001605] Using a transfer pipet, the contents of feeder vials were immediately transferred into 30 mL of warm CM2 in a 50 mL conical tube. The vial was washed with a small volume of CM2 to remove any residual cells in the vial and centrifuged at $400 \times$ CF for 5 minutes. Aspirated the supernatant and resuspended in 4 mL warm CM2 plus 3000 IU/mL IL-2. Removed 200 μ L for cell counting using the automated cell counter. Recorded the counts.

[001606] Resuspended cells at 1.3×10^7 cells/mL in warm CM2 plus 3000 IU/mL IL-2. Diluted TIL cells from 1.3×10^6 cells/mL to 1.3×10^5 cells/mL.

[001607] Setup Co-Culture. Diluted TIL cells from 1.3×10^6 cells/mL to 1.3×10^5 cells/mL. Added 4.5 mL of CM2 medium to a 15 mL conical tube. Removed TIL cells from incubator and resuspended well using a 10 mL serological pipet. Removed 0.5 mL of cells from the 1.3×10^6 cells/mL TIL suspension and added to the 4.5 mL of medium in the 15 mL conical tube. Returned TIL stock vial to incubator. Mixed well. Repeated for the second TIL line.

[001608] Transferred flasks with pre-warmed media for a single feeder lot from the incubator to the BSC. Mixed feeder cells by pipetting up and down several times with a 1 mL pipet tip and transferred 1 mL (1.3×10^7 cells) to each flask for that feeder lot. Added 60 μ L of OKT3 working stock (10 μ g/mL) to each flask. Returned the two control flasks to the incubator.

[001609] Transferred 1 mL (1.3×10^5) of each TIL lot to the correspondingly labeled T25 flask. Returned flasks to the incubator and incubate upright. Did not disturb until Day 5. This procedure was repeated for all feeder lots tested.

[001610] Day 5, Media change. Prepared CM2 with 3000 IU/mL IL-2. 10 mL is needed for each flask. With a 10 mL pipette, transferred 10 mL warm CM2 with 3000 IU/mL IL-2 to each flask. Returned flasks to the incubator and incubated upright until day 7. Repeated for all feeder lots tested.

[001611] Day 7, Harvest. Removed flasks from the incubator and transfer to the BSC, care as taken not to disturb the cell layer on the bottom of the flask. Without disturbing the cells growing on the bottom of the flasks, 10 mL of medium was removed from each test flask and 15 mL of medium from each of the control flasks.

[001612] Using a 10 mL serological pipet, the cells were resuspended in the remaining medium and mix well to break up any clumps of cells. After thoroughly mixing cell suspension by pipetting, removed 200 μ L for cell counting. Counted the TIL using the appropriate standard operating procedure in conjunction with the automatic cell counter equipment. Recorded counts in day 7. This procedure was repeated for all feeder lots tested.

[001613] Feeder control flasks were evaluated for replication incompetence and flasks containing TIL were evaluated for fold expansion from day 0.

[001614] Day 7, Continuation of Feeder Control Flasks to Day 14. After completing the day 7 counts of the feeder control flasks, 15 mL of fresh CM2 medium containing 3000 IU/mL

IL-2 was added to each of the control flasks. The control flasks were returned to the incubator and incubated in an upright position until day 14.

[001615] Day 14, Extended Non-proliferation of Feeder Control Flasks. Removed flasks from the incubator and transfer to the BSC, care was taken not to disturb the cell layer on the bottom of the flask. Without disturbing the cells growing on the bottom of the flasks, approximately 17 mL of medium was removed from each control flasks. Using a 5 mL serological pipet, the cells were resuspended in the remaining medium and mixed well to break up any clumps of cells. The volumes were recorded for each flask.

[001616] After thoroughly mixing the cell suspension by pipetting, 200 μ L was removed for cell counting. The TIL were counted using the appropriate standard operating procedure in conjunction with the automatic cell counter equipment and the counts were recorded. This procedure was repeated for all feeder lots tested.

B. Results and Acceptance Criteria Protocol

[001617] Results. The dose of gamma irradiation was sufficient to render the feeder cells replication incompetent. All lots were expected to meet the evaluation criteria and also demonstrated a reduction in the total viable number of feeder cells remaining on day 7 of the REP culture compared to day 0. All feeder lots were expected to meet the evaluation criteria of 100-fold expansion of TIL growth by day 7 of the REP culture. Day 14 counts of Feeder Control flasks were expected to continue the non-proliferative trend seen on day 7.

[001618] Acceptance Criteria. The following acceptance criteria were met for each replicate TIL line tested for each lot of feeder cells. Acceptance criteria were two-fold, as shown in Table 38 below.

TABLE 38. Embodiments of acceptance criteria.

Test	Acceptance criteria
Irradiation of MNC and Replication Incompetence	No growth observed at 7 and 14 days
TIL expansion	At least a 100-fold expansion of each TIL (minimum of 1.3×10^7 viable cells)

[001619] The dose of radiation was evaluated for its sufficiency to render the MNC feeder cells replication incompetent when cultured in the presence of 30 ng/mL OKT3 antibody and 3000 IU/mL IL-2. Replication incompetence was evaluated by total viable cell count (TVC) as determined by automated cell counting on day 7 and day 14 of the REP.

[001620] The acceptance criteria was “No Growth,” meaning the total viable cell number has not increased on day 7 and day 14 from the initial viable cell number put into culture on Day 0 of the REP.

[001621] The ability of the feeder cells to support TIL expansion was evaluated. TIL growth was measured in terms of fold expansion of viable cells from the onset of culture on day 0 of the REP to day 7 of the REP. On day 7, TIL cultures achieved a minimum of 100-fold expansion, (*i.e.*, greater than 100 times the number of total viable TIL cells put into culture on REP day 0), as evaluated by automated cell counting.

[001622] Contingency Testing of MNC Feeder Lots that do not meet acceptance criteria. In the event that an MNC feeder lot did not meet either of the acceptance criteria outlined above, the following steps will be taken to retest the lot to rule out simple experimenter error as its cause.

[001623] If there are two or more remaining satellite testing vials of the lot, then the lot was retested. If there were one or no remaining satellite testing vials of the lot, then the lot was failed according to the acceptance criteria listed above.

[001624] In order to be qualified, the lot in question and the control lot had to achieve the acceptance criteria above. Upon meeting these criteria, the lot is released for use.

EXAMPLE 4: PREPARATION OF IL-2 STOCK SOLUTION

[001625] 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.

[001626] Procedure. Prepared 0.2% Acetic Acid solution (HAc). Transferred 29 mL sterile water to a 50 mL conical tube. Added 1 mL 1N 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.

[001627] 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.

[001628] 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).

[001629] Calculated the volume of 1% HSA required for rhIL-2 lot by using the equation below:

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

[001630] For example, according to the COA of rhIL-2 lot 10200121 (Cellgenix), 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.

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

[001631] 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.

[001632] Storage of rhIL-2 solution. For short-term storage (<72hrs), stored vial at 4°C. For long-term storage (>72hrs), aliquoted vial into smaller volumes and stored in cryovials at -20°C

until ready to use. Avoided freeze/thaw cycles. Expired 6 months after date of preparation. Rh-IL-2 labels included vendor and catalog number, lot number, expiration date, operator initials, concentration and volume of aliquot.

EXAMPLE 5: CRYOPRESERVATION PROCESS

[001633] This example describes a cryopreservation process method for TILs prepared with the procedures described herein using the CryoMed Controlled Rate Freezer, Model 7454 (Thermo Scientific).

[001634] 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).

[001635] 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 6: EXEMPLARY EMBODIMENT OF THE PROCESS FOR ENRICHING TUMOR REACTIVE TILS USING AUTOLOGOUS TUMOR DIGEST

[001636] Fig. 1 illustrates an embodiment of the processes for enriching tumor reactive TILs using autologous tumor digest.

Tumor Preparation

[001637] A tumor sample freshly resected from a cancer patient is fragmented into approximately 2-6-mm³ fragments, and randomly distributed to provide material for: (1) production of pre-REP TIL product; and (2) cryopreservation of autologous tumor digest, both of which can proceed independently and simultaneously.

[001638] Tumor Processing. Obtain tumor specimen and transfer into suite at 2-8 °C immediately for processing. Aliquot tumor wash media. Tumor wash 1 is performed using 8" forceps (W3009771). The tumor is removed from the specimen bottle and transferred to the

“Wash 1” dish prepared. This is followed by tumor wash 2 and tumor wash 3. Measure and assess tumor. Assess whether > 30% of entire tumor area observed to be necrotic and/or fatty tissue. Clean up dissection if applicable. If tumor is large and >30% of tissue exterior is observed to be necrotic/fatty, perform “clean up dissection” by removing necrotic/fatty tissue while preserving tumor inner structure using a combination of scalpel and/or forceps. Dissect tumor. Using a combination of scalpel and/or forceps, cut the tumor specimen into even, appropriately sized fragments (up to 6 intermediate fragments). Transfer intermediate tumor fragments. Dissect tumor fragments into pieces approximately 3x3x3mm in size. Store Intermediate Fragments to prevent drying. Repeat intermediate fragment dissection. Determine number of pieces collected. If desirable tissue remains, select additional favorable tumor pieces from the “favorable intermediate fragments” 6-well plate to fill the drops for a maximum of 50 pieces.

Production of PreREP TIL Product

[001639] Day 0

[001640] CM1 Media Preparation. In a biological safety cabinet (BSC) add reagents to RPMI 1640 Media bottle. Add 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).

[001641] Remove unnecessary materials from BSC. Pass out media reagents from BSC, leave Gentamicin Sulfate and HBSS in BSC for Formulated Wash Media preparation.

[001642] Thaw IL-2 aliquot. Thaw one 1.1 mL IL-2 aliquot (6×10^6 IU/mL) (BR71424) until all ice has melted. Record IL-2: Lot # and Expiry.

[001643] 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. Add CM1 Day 0 Media 1 bottle and IL-2 (6×10^6 IU/mL) 1.0 mL.

[001644] Pass G-REX100MCS into BSC. Aseptically pass G-REX100MCS (W3013130) into the BSC.

[001645] Pump all Complete CM1 Day 0 Media into G-REX100MCS flask. Tissue Fragments Conical or GRex100MCS.

[001646] Tumor Wash Media Preparation. In the BSC, add 5.0 mL Gentamicin (W3009832 or W3012735) to 1 x 500 mL HBSS Media (W3013128) bottle. Add per bottle: HBSS (500.0 mL); Gentamicin sulfate, 50 mg/mL (5.0 mL). Filter HBSS containing gentamicin prepared through a 1L 0.22-micron filter unit (W1218810).

[001647] Prepared conical tube. Transfer tumor pieces to a 50 mL conical tube. Prepare BSC for G-REX100MCS. Remove G-REX100MCS from incubator. Aseptically pass G-REX100MCS flask into the BSC. Add tumor fragments to G-REX100MCS flask. Evenly distributed pieces.

[001648] 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.

[001649] The pre-REP step is performed by culturing ≤ 50 tumor fragments in a G-REX-100MCS flask in the presence of CM1 with 6000 IU/mL IL-2 for 6–9-days.

[001650] After process is complete, discard any remaining warmed media and thawed aliquots of IL-2.

[001651] TIL Harvest. Preprocessing table. Incubator parameters: Temperature LED display: 37.0 ± 2.0 °C; CO₂ Percentage: 5.0 ± 1.5 % CO₂. Remove G-REX100MCS from incubator. Prepare 300 mL Transfer Pack. Weld transfer packs to G-REX100MCS.

[001652] Prepare flask for TIL Harvest and initiation of TIL Harvest. Using the GatheRex, transfer the cell suspension through the blood filter into the 300 mL transfer pack. Inspect membrane for adherent cells.

[001653] Rinse flask membrane. Close clamps on G-REX100MCS. Ensure all clamps are closed. Heat seal the TIL and the “Supernatant” transfer pack. Calculate volume of TIL suspension. Prepare Supernatant Transfer Pack for Sampling.

[001654] Incubate TIL. Place TIL transfer pack in incubator until needed. Perform cell counts and calculations. Determine the Average of Viable Cell Concentration and Viability of the cell counts performed. $\text{Viability} \div 2$. $\text{Viable Cell Concentration} \div 2$. Determine Upper and Lower Limit for counts. Lower Limit: $\text{Average of Viable Cell Concentration} \times 0.9$. Upper

Limit: Average of Viable Cell Concentration $\times 1.1$. Confirm both counts within acceptable limits. Determine an average Viable Cell Concentration from all four counts performed.

[001655] The procedures for obtaining cell and viability counts use the Nexcelom Cellometer K2 or equivalent cell counter.

[001656] Adjust Volume of TIL Suspension: Calculate the adjusted volume of TIL suspension after removal of cell count samples. Total TIL Cell Volume (A). Volume of Cell Count Sample Removed (4.0 mL) (B) Adjusted Total TIL Cell Volume $C=A-B$.

[001657] Calculate Total Viable TIL Cells. Average Viable Cell Concentration*: Total Volume; Total Viable Cells: $C = A \times B$.

Tumor Processing and Digestion

[001658] At the same time or after the initiation of the preREP process, start tumor processing and digestion.

[001659] If using GentleMACS OctoDissociator, transfer the tumor fragments to a GentleMACS C-Tube (C-tube) or 50-ml conical tube in the 5-ml of digest cocktail (in HBSS) indicated above. Transfer 2-3 fragments (4-6mm) to each C-tube.

[001660] Transfer each C-tube (Miltenyi Biotec, Germany, 130-096-334) to the GentleMACS OctoDissociator (Miltenyi Biotec, Germany, 130-095-937). Use according to the manufacturer's directions. Note, each tumor histology has a recommended program for tumor dissociation. Select the appropriate program for the respective tumor histology. The dissociation would approximately one hour.

[001661] If the GentleMACS OctoDissociator is not available, use a standard rotator. Placed 2-3 tumor fragments in a 50-ml conical tube (sealed with parafilm to avoid leakage) and secure to the rotator. Place the rotator, at 37°C, 5% CO₂ humidified incubator on constant rotation for 1-2 hours. Alternatively, the tumor fragments could be digested at RT overnight, also with constant rotation.

[001662] Post-digest, remove the C-tube from the Octodissociator or rotator. Attach a 0.22- μ m strainer to sterile Falcon conical tube. Using a pipette, pass all contents from the C-tube/ or 50-ml conical (5ml) through the 0.22- μ m strainer into a 50-ml conical. Wash the C-tube/50-ml

conical with 10-ml of HBSS and apply to the strainer. Use the flat end of a sterile syringe plunger to dissociate any remaining non-digested tumor through the filter. Add CM1 or HBSS up to 50-ml and cap the tube.

[001663] Pellet the samples by centrifugation, 1500 rpm, 5 min at RT (with an acceleration and deceleration of 9). Carefully remove the liquid, resuspended pellet in 5-ml of CM1 for cell counting and viability assessment.

[001664] Put aside whole tumor digest for the following: 1. Cell culture (unselected TIL control) 2. FMO flow cytometry controls 3. Pre-sort whole tumor digest phenotyping assays 4. Frozen for tumor reactivity/cell killing assays. The number of cells put aside will depend on the total digest yield and tumor histology.

Enzyme Preparation for Tumor Digestion (Using Research Grade DNase, Collagenase and Hyaluronidase)

[001665] Reconstitute the lyophilized enzymes in the amount of sterile HBSS indicated for each of the digestion enzymes below. These enzymes are prepared as 10X. Pipette up and down several times and swirl to ensure complete reconstitution.

[001666] Reconstitute 1-g of Collagenase IV (Sigma, MO, C5138) in 10-ml HBSS (to make a 100-mg/mL stock). Mix by pipetting up and down to dissolve. If not dissolved after reconstitution, place in a 37°C H₂O bath for 5 minutes. Aliquot into 1-ml vials. This is the 100-mg/mL 10X working stock for collagenase.

[001667] Prepare the DNase (Sigma, MO, D5025) stock solution (10,000-IU/mL). The units of DNase for each lot is provided in the accompanying data sheet. Calculate the appropriate volume of HBSS to reconstitute the 100-mg lyophilized DNase stock. For example, if the DNase stock is 2000-U/mg, the total DNase in the stock is 200,000-IU (2000-IU/mg X 100-mg). Dilute to a working stock of 10,000IU, add 20-ml of HBSS to the 100mg of DNase (200,000IU/20ml=10,000U/mL). Aliquot into 1-ml vials. This is the 10,000IU/mL 10X working stock for DNase.

[001668] Prepare the hyaluronidase 10-mg/mL (Sigma, MO, H2126) stock solution. Reconstitute the 500-mg vial with 50-ml of HBSS to make a 10-mg/mL stock solution. Aliquot into 1-ml vials. This was the 10-mg/mL 10X working stock for hyaluronidase.

[001669] Dilute the stock digest enzymes to 1X. To make a 1X working solution, add 500-ml each of the collagenase, DNase and hyaluronidase to 3.5-ml of HBSS. Add the digest cocktail directly to the C-tube.

Enzyme Preparation for Tumor Digestion (using GMP Collagenase and Neutral Protease)

[001670] Reconstitute the lyophilized enzymes in the amount of sterile HBSS indicated for each of the digestion enzymes below. Be sure to capture any residual powder from the sides of the bottles and from the protective foil on the bottles opening. Pipette up and down several times and swirl to ensure complete reconstitution.

[001671] Reconstitute the Collagenase AF-1 (Nordmark, Sweden, N0003554) in 10-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 2892 PZ U/vial. After reconstitution the collagenase stock was 289.2 PZ U/mL.

[001672] Reconstitute the Neutral protease (Nordmark, Sweden, N0003553) in 1-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 175 DMC U/vial. Therefore, after reconstitution the neutral protease stock was 175 DMC/mL.

[001673] Reconstitute the DNase I (Roche, Switzerland, 03724751) in 1-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 4KU/vial. Therefore, after reconstitution the DNase stock is 4KU/vial.

[001674] Prepare the working GMP digest cocktail. Add 10.2- μ l of the neutral protease (0.36 DMC U/mL), 21.3- μ l of collagenase AF-1 (1.2 PZ/mL) and 250- μ l of DNase I (200 U/mL) to 4.7-ml of sterile HBSS. Place the digest cocktail directly into the C-tube.

Cleaning up the Digest using the Debris Removal Kit

[001675] Debris could be removed from the tumor digest using the Debris Removal Solution (Miltenyi Biotec, Germany, Cat#130-109-398) or other equivalent reagent, according to the manufacturer's directions.

[001676] Centrifuge the tumor cell suspension at 300Xg for 10 minutes at 4oC and aspirate supernatant completely.

[001677] Resuspend cell suspension carefully with the appropriate volume of cold buffer according to the table below and transfer the cell suspension to a 15ml conical tube. DO NOT VORTEX.

	<u>Resuspension (PBS)</u>	<u>Debris Removal Solution</u>	<u>Overlay (PBS)</u>
0.5-1g tissue	6200- μ l	1800- μ l	4-ml
> 0.5 g tissue	3100- μ l	900- μ l	4-ml

[001678] Add appropriate volume of cold Debris Removal Solution and mix well by pipetting slowly up and down 10-20 times using a 5-ml pipette. Overlayed very gently with 4-ml of cold buffer. Tilt the tube and pipette very slowly to ensure that the PBS/D-PBS phase overlays the cell suspension and phases are not mixed. Centrifuge the tumor cell suspension at 3000Xg for 10 minutes at 4°C with full acceleration and full break. Three phases should form. Aspirate the two top phases completely and discard them.

[001679] The bottom phase contains the Debris Removal Solution and the cells. Be sure to leave at least as much volume at the bottom as was added of the Debris Removal solution. (i.e. if 1ml of solution was added leave at least 1-ml at the bottom of the tube).

[001680] Bring up to 15-ml with cold buffer and invert the tube at least three times. DO NOT VORTEX. Centrifuge at 4°C and 1000Xg for 10 minutes with full acceleration and full break. Resuspend cells in HBSS or media for cell count.

Co-Culture PreREP TIL Product with Autologous Tumor Digest

[001681] Add the cleaned-up tumor digest to the pre-REP culture media and allow the co-culture of pre-REP TIL product and the tumor digest to proceed for 24-72 hours. In some embodiments, the residual tumor fragments will be removed from the pre-REP cell culture medium before co-culturing with the autologous tumor digest.

Staining Co-Cultured TILs for Cell Sorting

[001682] The co-cultured TILs are stained with a cocktail that includes anti-4-1BB, and anti-OX40 antibodies according to the following protocol. Post-co-culture, resuspend the TILs in 10-ml HBSS.

[001683] Resuspend pellet in FACS buffer (1 X HBSS, 1mM EDTA, 2% fetal bovine serum). The amount of FACS buffer added to the pellet is based upon the size of the pellet. The staining volume should be about 3 times the size of the pellet. Therefore, if there is 300- μ l of cells, the volume of buffer should be at least 900- μ l.

[001684] For antibody addition, each 100- μ l of volume is equivalent to one test (titered amount of antibody). *I.e.*, if there is 1-ml of volume, 10X the amount of titered antibody is required. Add a titered amount of each of the following antibodies; anti-4-1BB-PE-Cy7, and anti-OX40 FITC per 100- μ l of volume. Incubate cells on ice for 30 minutes. Protect from light during incubation. Agitate a couple times during incubation. Resuspend cells in 20-ml of FACS buffer. Pass solution through a 70-micron cell strainer into a new 50-ml conical. Centrifuge, 400Xg, 5 min at RT (acceleration and deceleration of 9). Aspirate. Resuspend cells in up to 10e6/mL TOTAL (live+dead) in FACS buffer. Minimum volume is 300- μ l. Transfer to sterile polypropylene FACS tubes or 15-ml conical tubes. 3-ml/tube for FACS sorting. Prepare 15-ml collection tubes for the sorted populations. Place 2-ml of FACS buffer in the tubes.

Collection of Tumor Reactive TILs

[001685] To assess for reactivity of the TILs after the co-culture, 1.0 mL of the supernatant from the co-culture is assayed for IFN- γ level.

[001686] The co-cultured TILs are stained with anti-4-1BB and anti-OX40 antibodies for FACS sorting. After staining, the TILs are sorted using a SONY FX 500 cytometer by 2-color sorting. 4-1BB, OX40 double positive TILs are collected as a population of tumor reactive TILs.

REP of Tumor Reactive TILs

[001687] The collected tumor reactive TILs are further expanded using REP conditions for 11 days.

[001688] Day 0

[001689] Prepare CM2 and place at 4 °C.

[001690] Prepare G-Rex 500MCS Flask. Using 10 mL syringe aseptically transfer 0.5mL of IL-2 (stock is 6×10^6 IU/mL) for each liter of CM2 into the bioprocess bag through an unused sterile female luer connector. Open exterior packaging and place G-Rex 500MCS in the BSC.

Close all clamps on the device except large filter line. Sterile weld the red harvest line from the G-Rex 500MCS to the pump tubing outlet line. Connect bioprocess bag female luer to male luer of the Pump boot. Hang the bioprocess bag on the IV pole, open the clamps and pump 4.5 Liters of the CM2 media into the G-Rex 500MCS. Clear the line, clamp, and heat seal. Place G-Rex 500MCS in the incubator.

[001691] Feeder Cells. Record the dry weight of a 1L transfer pack (TP). Pump 500mL CM2 by weight into the TP. Verify and Log out feeder cell bags. Thaw feeder cells in the 37° C (+/- 1° C) water bath. Record temperature of water bath. Dispense feeder cells into the TP using a syringe. Heat seal the TP and place in incubator. Perform a cell count on the feeder cell sample and calculate the total viable cell density in the TP.

[001692] Using a 1mL syringe and 18G needle draw up OKT3, transfer to the feeder TP through the female luer. Sterile weld the feeder bag to the red line on the G-Rex 500MCS. Unclamp the line and allow the feeder cells to flow into the flask by gravity.

[001693] Added tumor reactive TILs to G-REX500MCS. Heat seal. Incubate G-REX500MCS at 37.0±2.0 °C, CO₂ Percentage: 5.0±1.5 %CO₂.

[001694]

[001695] Day 5

[001696] Media Preparation. Warm one 10L bag of CM4 in a 37°C incubator at least 30 minutes or until ready to use. Remove the G-Rex 500MCS flask from the incubator and place on the benchtop adjacent the GatheRex. Check all clamps are closed except large filter line. Move the clamp on the quick connect line close to the “T” junction. Heat seal a 2L transfer pack 2” below the “Y removing the spike and record dry weight. Sterile weld the 2L TP to the clear collection line on the G-Rex 500MCS. After removal of the supernatant, swirl the flask until all the cells have been detached from the membrane. Release all clamps leading to the 2L TP and using the GatheRex transfer the residual cell suspension into the 2L TP.

[001697] Perform single cell counts and record data and attach counting raw data to batch record. Document Dilution. Document the Cellometer counting program. Verify the correct dilution is entered into the Cellometer.

[001698] Attach an 18G needle to a 10mL syringe and draw 5mL of IL-2 into the syringe (final concentration is 3000 IU/ml). Remove the needle and aseptically attach the syringe to the plasma transfer set and dispensed IL-2 into a 10L bag of Aim V with Glutamax.

[001699] Prepare G-REX500MCS Flasks. Determine amount of CM4 to add to flasks. Record volume of cells added per flask and volume of CM4 5000mL-A. Pump determined amount of CM4 into the G-Rex 500MCS using lines on flask as guide. Split the TILs from the G-REX500MCS and add to the flasks.

[001700] Monitor Incubator parameters: Temperature LED display: 37.0 ± 2.0 °C, CO₂ Percentage: 5.0 ± 1.5 %CO₂. Remove G-REX500MCS from the incubator. Prepare and label 1 L Transfer Pack as TIL Suspension and weigh 1L.

[001701] Day 11

[001702] Close all clamps on a 10 L Labtainerbag. Attach Baxter extension set to the 10L bag via luer connection. Sterile weld the red media removal line from the G-Rex 500MCS to the extension set on the 10L bioprocess bag. Sterile weld the clear cell removal line from the G-Rex 500MCS to a 3L collection bag and label "pooled cell suspension". Using the Gatherex pump, volume reduce the first flask.

[001703] Close the clamp on the supernatant bag and red line. Swirl the G-REX 500M flask until the TILs are completely resuspended while avoiding splashing or foaming. Make sure all cells have been dislodged from the membrane. Open clamps on clear line and 3L cell bag. Start Gatherex to collect the cell suspension. Close clamp on the line to the cell collection bag. Release clamps on Gatherex.

[001704] Repeat the above steps for each flask.

[001705]

EXAMPLE 7: EXEMPLARY EMBODIMENT OF THE PROCESS FOR ENRICHING TUMOR REACTIVE TILS USING AUTOLOGOUS DC OR DC LIKE CELLS

[001706] Fig. 2 illustrates an embodiment of the processes for enriching tumor reactive TILs using autologous dendritic cells (DCs) or DC like cells using the protocols of Example 6.

Tumor Preparation

[001707] A tumor sample freshly resected from a cancer patient is fragmented into approximately 2-6-mm³ fragments, and randomly distributed to provide material for: (1) production of pre-REP TIL product; and (2) cryopreservation of autologous tumor digest, both of which can proceed independently and simultaneously.

[001708] Tumor Processing. Obtain tumor specimen and transfer into suite at 2-8 °C immediately for processing. Aliquot tumor wash media. Tumor wash 1 is performed using 8” forceps (W3009771). The tumor is removed from the specimen bottle and transferred to the “Wash 1” dish prepared. This is followed by tumor wash 2 and tumor wash 3. Measure and assess tumor. Assess whether > 30% of entire tumor area observed to be necrotic and/or fatty tissue. Clean up dissection if applicable. If tumor is large and >30% of tissue exterior is observed to be necrotic/fatty, perform “clean up dissection” by removing necrotic/fatty tissue while preserving tumor inner structure using a combination of scalpel and/or forceps. Dissect tumor. Using a combination of scalpel and/or forceps, cut the tumor specimen into even, appropriately sized fragments (up to 6 intermediate fragments). Transfer intermediate tumor fragments. Dissect tumor fragments into pieces approximately 3x3x3mm in size. Store Intermediate Fragments to prevent drying. Repeat intermediate fragment dissection. Determine number of pieces collected. If desirable tissue remains, select additional favorable tumor pieces from the “favorable intermediate fragments” 6-well plate to fill the drops for a maximum of 50 pieces.

Production of PreREP TIL Product

[001709] Day 0

[001710] CM1 Media Preparation. In a biological safety cabinet (BSC) add reagents to RPMI 1640 Media bottle. Add 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).

[001711] Remove unnecessary materials from BSC. Pass out media reagents from BSC, leave Gentamicin Sulfate and HBSS in BSC for Formulated Wash Media preparation.

[001712] Thaw IL-2 aliquot. Thaw one 1.1 mL IL-2 aliquot (6x10⁶ IU/mL) (BR71424) until all ice has melted. Record IL-2: Lot # and Expiry.

[001713] 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. Add CM1 Day 0 Media 1 bottle and IL-2 (6x10⁶ IU/mL) 1.0 mL.

[001714] Pass G-REX100MCS into BSC. Aseptically pass G-REX100MCS (W3013130) into the BSC.

[001715] Pump all Complete CM1 Day 0 Media into G-REX100MCS flask. Tissue Fragments Conical or GRex100MCS.

[001716] Tumor Wash Media Preparation. In the BSC, add 5.0 mL Gentamicin (W3009832 or W3012735) to 1 x 500 mL HBSS Media (W3013128) bottle. Add per bottle: HBSS (500.0 mL); Gentamicin sulfate, 50 mg/mL (5.0 mL). Filter HBSS containing gentamicin prepared through a 1L 0.22-micron filter unit (W1218810).

[001717] Prepared conical tube. Transfer tumor pieces to a 50 mL conical tube. Prepare BSC for G-REX100MCS. Remove G-REX100MCS from incubator. Aseptically pass G-REX100MCS flask into the BSC. Add tumor fragments to G-REX100MCS flask. Evenly distributed pieces.

[001718] 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.

[001719] The pre-REP step is performed by culturing ≤ 50 tumor fragments in a G-REX-100MCS flask in the presence of CM1 with 6000 IU/mL IL-2 for 6–9-days.

[001720] After process is complete, discard any remaining warmed media and thawed aliquots of IL-2.

[001721] TIL Harvest. Preprocessing table. Incubator parameters: Temperature LED display: 37.0±2.0 °C; CO₂ Percentage: 5.0±1.5 % CO₂. Remove G-REX100MCS from incubator. Prepare 300 mL Transfer Pack. Weld transfer packs to G-REX100MCS.

[001722] Prepare flask for TIL Harvest and initiation of TIL Harvest. Using the GatheRex, transfer the cell suspension through the blood filter into the 300 mL transfer pack. Inspect membrane for adherent cells.

[001723] Rinse flask membrane. Close clamps on G-REX100MCS. Ensure all clamps are closed. Heat seal the TIL and the “Supernatant” transfer pack. Calculate volume of TIL suspension. Prepare Supernatant Transfer Pack for Sampling.

[001724] Incubate TIL. Place TIL transfer pack in incubator until needed. Perform cell counts and calculations. Determine the Average of Viable Cell Concentration and Viability of the cell counts performed. $\text{Viability} \div 2$. $\text{Viable Cell Concentration} \div 2$. Determine Upper and Lower Limit for counts. Lower Limit: $\text{Average of Viable Cell Concentration} \times 0.9$. Upper Limit: $\text{Average of Viable Cell Concentration} \times 1.1$. Confirm both counts within acceptable limits. Determine an average Viable Cell Concentration from all four counts performed.

[001725] The procedures for obtaining cell and viability counts use the Nexcelom Cellometer K2 or equivalent cell counter.

[001726] Adjust Volume of TIL Suspension: Calculate the adjusted volume of TIL suspension after removal of cell count samples. Total TIL Cell Volume (A). Volume of Cell Count Sample Removed (4.0 mL) (B) Adjusted Total TIL Cell Volume $C=A-B$.

[001727] Calculate Total Viable TIL Cells. $\text{Average Viable Cell Concentration}^* \times \text{Total Volume}$; Total Viable Cells: $C = A \times B$.

Tumor Processing and Digestion

[001728] At the same time or after the initiation of the preREP process, start tumor processing and digestion.

[001729] If using GentleMACS OctoDissociator, transfer the tumor fragments to a GentleMACS C-Tube (C-tube) or 50-ml conical tube in the 5-ml of digest cocktail (in HBSS) indicated above. Transfer 2-3 fragments (4-6mm) to each C-tube.

[001730] Transfer each C-tube (Miltenyi Biotec, Germany, 130-096-334) to the GentleMACS OctoDissociator (Miltenyi Biotec, Germany, 130-095-937). Use according to the manufacturer's directions. Note, each tumor histology has a recommended program for tumor dissociation. Select the appropriate program for the respective tumor histology. The dissociation would approximately one hour.

[001731] If the GentleMACS OctoDissociator is not available, use a standard rotator. Placed 2-3 tumor fragments in a 50-ml conical tube (sealed with parafilm to avoid leakage) and secure to the rotator. Place the rotator, at 37°C, 5% CO₂ humidified incubator on constant rotation for 1-2 hours. Alternatively, the tumor fragments could be digested at RT overnight, also with constant rotation.

[001732] Post-digest, remove the C-tube from the Octodissociator or rotator. Attach a 0.22- μ m strainer to sterile Falcon conical tube. Using a pipette, pass all contents from the C-tube/ or 50-ml conical (5ml) through the 0.22- μ m strainer into a 50-ml conical. Wash the C-tube/50-ml conical with 10-ml of HBSS and apply to the strainer. Use the flat end of a sterile syringe plunger to dissociate any remaining non-digested tumor through the filter. Add CM1 or HBSS up to 50-ml and cap the tube.

[001733] Pellet the samples by centrifugation, 1500 rpm, 5 min at RT (with an acceleration and deceleration of 9). Carefully remove the liquid, resuspended pellet in 5-ml of CM1 for cell counting and viability assessment.

[001734] Put aside whole tumor digest for the following: 1. Cell culture (unselected TIL control) 2. FMO flow cytometry controls 3. Pre-sort whole tumor digest phenotyping assays 4. Frozen for tumor reactivity/cell killing assays. The number of cells put aside will depend on the total digest yield and tumor histology.

Enzyme Preparation for Tumor Digestion (Using Research Grade DNase, Collagenase and Hyaluronidase)

[001735] Reconstitute the lyophilized enzymes in the amount of sterile HBSS indicated for each of the digestion enzymes below. These enzymes are prepared as 10X. Pipette up and down several times and swirl to ensure complete reconstitution.

[001736] Reconstitute 1-g of Collagenase IV (Sigma, MO, C5138) in 10-ml HBSS (to make a 100-mg/mL stock). Mix by pipetting up and down to dissolve. If not dissolved after reconstitution, place in a 37°C H₂O bath for 5 minutes. Aliquot into 1-ml vials. This is the 100-mg/mL 10X working stock for collagenase.

[001737] Prepare the DNase (Sigma, MO, D5025) stock solution (10,000-IU/mL). The units of DNase for each lot is provided in the accompanying data sheet. Calculate the

appropriate volume of HBSS to reconstitute the 100-mg lyophilized DNase stock. For example, if the DNase stock is 2000-U/mg, the total DNase in the stock is 200,000-IU (2000-IU/mg X 100-mg). Dilute to a working stock of 10,000IU, add 20-ml of HBSS to the 100mg of DNase (200,000IU/20ml=10,000U/mL). Aliquot into 1-ml vials. This is the 10,000IU/mL 10X working stock for DNase.

[001738] Prepare the hyaluronidase 10-mg/mL (Sigma, MO, H2126) stock solution. Reconstitute the 500-mg vial with 50-ml of HBSS to make a 10-mg/mL stock solution. Aliquot into 1-ml vials. This was the 10-mg/mL 10X working stock for hyaluronidase.

[001739] Dilute the stock digest enzymes to 1X. To make a 1X working solution, add 500-ml each of the collagenase, DNase and hyaluronidase to 3.5-ml of HBSS. Add the digest cocktail directly to the C-tube.

Enzyme Preparation for Tumor Digestion (using GMP Collagenase and Neutral Protease)

[001740] Reconstitute the lyophilized enzymes in the amount of sterile HBSS indicated for each of the digestion enzymes below. Be sure to capture any residual powder from the sides of the bottles and from the protective foil on the bottles opening. Pipette up and down several times and swirl to ensure complete reconstitution.

[001741] Reconstitute the Collagenase AF-1 (Nordmark, Sweden, N0003554) in 10-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 2892 PZ U/vial. After reconstitution the collagenase stock was 289.2 PZ U/mL.

[001742] Reconstitute the Neutral protease (Nordmark, Sweden, N0003553) in 1-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 175 DMC U/vial. Therefore, after reconstitution the neutral protease stock was 175 DMC/mL.

[001743] Reconstitute the DNase I (Roche, Switzerland, 03724751) in 1-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 4KU/vial. Therefore, after reconstitution the DNase stock is 4KU/vial.

[001744] Prepare the working GMP digest cocktail. Add 10.2- μ l of the neutral protease (0.36 DMC U/mL), 21.3- μ l of collagenase AF-1 (1.2 PZ/mL) and 250- μ l of DNase I (200 U/mL) to 4.7-ml of sterile HBSS. Place the digest cocktail directly into the C-tube.

Cleaning up the Digest using the Debris Removal Kit

[001745] Debris could be removed from the tumor digest using the Debris Removal Solution (Miltenyi Biotec, Germany, Cat#130-109-398) or other equivalent reagent, according to the manufacturer's directions.

[001746] Centrifuge the tumor cell suspension at 300Xg for 10 minutes at 4°C and aspirate supernatant completely.

[001747] Resuspend cell suspension carefully with the appropriate volume of cold buffer according to the table below and transfer the cell suspension to a 15ml conical tube. DO NOT VORTEX.

	<u>Resuspension (PBS)</u>	<u>Debris Removal Solution</u>	<u>Overlay (PBS)</u>
0.5-1g tissue	6200- µl	1800- µl	4-ml
> 0.5 g tissue	3100- µl	900- µl	4-ml

[001748] Add appropriate volume of cold Debris Removal Solution and mix well by pipetting slowly up and down 10-20 times using a 5-ml pipette. Overlayed very gently with 4-ml of cold buffer. Tilt the tube and pipette very slowly to ensure that the PBS/D-PBS phase overlays the cell suspension and phases are not mixed. Centrifuge the tumor cell suspension at 3000Xg for 10 minutes at 4°C with full acceleration and full break. Three phases should form. Aspirate the two top phases completely and discard them.

[001749] The bottom phase contains the Debris Removal Solution and the cells. Be sure to leave at least as much volume at the bottom as was added of the Debris Removal solution. (i.e. if 1ml of solution was added leave at least 1-ml at the bottom of the tube).

[001750] Bring up to 15-ml with cold buffer and invert the tube at least three times. DO NOT VORTEX. Centrifuge at 4°C and 1000Xg for 10 minutes with full acceleration and full break. Resuspend cells in HBSS or media for cell count.

Tumor Peptide generation

[001751] In some embodiments, tumor peptide can be generated from tumor fragments using the following protocol:

[001752] Homogenize tumor fragments into fine pieces.

[001753] Add 50-fold of extraction buffer, based upon estimated volume or wet weight. The amount can be adjusted based upon protein concentration. Use BCA Protein Assay (ThermoFisher) or Bradford assay for protein estimation.

[001754] Dissolve protein pellet in the minimum volume necessary of 8M Urea/2M Thiourea/400mM Ammonium Biocarbonate (Ambic). Volume should be no more than 50-100 uL+ protease inhibitor.

[001755] Incubate on ice for 30 min with frequent vortexing.

[001756] Add Dithiothreitol (DTT) to 5mM. Pipette 5uL of the DTT solution into the protein solution if your volume is 100uL, 2.5uL if volume is 50uL.

[001757] Incubate solution at 50 °C for 30 min.

[001758] Allow the solution to cool to room temp and add Iodoacetemide (IAA) to 12.5mM. Pipette 12uL of the IAA solution to the protein solution if volume is 100uL, 6uL if volume is 50uL. Incubate the solution at room temperature in the dark for 20 min.

[001759] Quench the reaction by adding another 5uL DTT to the solution, 2.5uL if 50uL volume.

[001760] Dilute solution 8x with 50mM Ambic so final concentrations are 1M Urea/250mM Thiourea/50mM Ambic.

[001761] Add trypsin 1:50 (trypsin to protein). If protein concentration is not known add 100ng of trypsin solution in 50mM Ambic. Incubate overnight at 37°C.

[001762] Acidify the solution to inactivate Trypsin by adding Formic acid to 0.5% final concentration.

[001763] Desalt peptides with Ziptip Pipette tips.

Mature DC generation

[001764] Apheresis or blood is obtained from the cancer patient and processed to obtain PBMCs. PBMCs are incubated in a flask for approximately 3-4 hours to allow the monocytes to adhere, and the non-adherent cells are discarded by gently tilting the flask and changing the media without disturbing the attached monocytes. Monocytes are then cultured in the presence of 250 IU/ml of GM-CSF and 800 IU/ml of IL-4 for 24-hours. The following day, the media will be

changed and supplied with 2X 250 IU/ml of GM-CSF and 800 IU/ml of IL-4 for another 5 days. After this the monocytes are differentiated into immature DCs.

[001765] Tumor peptides are generated from the cleaned-up tumor digest using the protocol above. The immature DCs are then pulsed with the tumor lysate or tumor peptides for 16-24 hours. The pulsed DCs are then cultured in the presence of 2000 IU/ml of TNF α , 200 IU/ml of IL-6, and 400 IU/ml of IL-1 β for about 24 hours to generate mature DCs.

Co-Culture with Autologous DC

[001766] Add the mature DCs to the pre-REP culture media at a ratio of 2:1 or 10:1 TIL:DC and allow the co-culture of pre-REP'd TILs and the mature DCs to proceed for 24-72 hours. In some embodiments, the residual tumor fragments will be removed from the pre-REP cell culture medium before co-culturing with the mature DCs.

Staining Co-Cultured TILs for Cell Sorting

[001767] The co-cultured TILs are stained with a cocktail that includes anti-4-1BB, and anti-OX40 antibodies according to the following protocol. Post-co-culture, resuspend the TILs in 10-ml HBSS.

[001768] Resuspend pellet in FACS buffer (1 X HBSS, 1mM EDTA, 2% fetal bovine serum). The amount of FACS buffer added to the pellet is based upon the size of the pellet. The staining volume should be about 3 times the size of the pellet. Therefore, if there is 300- μ l of cells, the volume of buffer should be at least 900- μ l.

[001769] For antibody addition, each 100- μ l of volume is equivalent to one test (titered amount of antibody). *I.e.*, if there is 1-ml of volume, 10X the amount of titered antibody is required. Add a titered amount of each of the following antibodies; anti-4-1BB-PE-Cy7, and anti-OX40 FITC per 100- μ l of volume. Incubate cells on ice for 30 minutes. Protect from light during incubation. Agitate a couple times during incubation. Resuspend cells in 20-ml of FACS buffer. Pass solution through a 70-micron cell strainer into a new 50-ml conical. Centrifuge, 400Xg, 5 min at RT (acceleration and deceleration of 9). Aspirate. Resuspend cells in up to 10e6/mL TOTAL (live+dead) in FACS buffer. Minimum volume is 300- μ l. Transfer to sterile polypropylene FACS tubes or 15-ml conical tubes. 3-ml/tube for FACS sorting. Prepare 15-ml collection tubes for the sorted populations. Place 2-ml of FACS buffer in the tubes.

Collection of Tumor Reactive TILs

[001770] To assess for reactivity of the TILs after the co-culture, 1.0 mL of the supernatant from the co-culture is assayed for IFN- γ level.

[001771] The co-cultured TILs are stained with anti-4-1BB and anti-OX40 antibodies for FACS sorting. After staining, the TILs are sorted using a SONY FX 500 cytometer by 2-color sorting. 4-1BB, OX40 double positive TILs are collected as a population of tumor reactive TILs.

REP of Tumor Reactive TILs

[001772] The collected tumor reactive TILs are further expanded using REP conditions for 11 days.

[001773] Day 0

[001774] Prepare CM2 and place at 4 °C.

[001775] Prepare G-Rex 500MCS Flask. Using 10 mL syringe aseptically transfer 0.5mL of IL-2 (stock is 6×10^6 IU/mL) for each liter of CM2 into the bioprocess bag through an unused sterile female luer connector. Open exterior packaging and place G-Rex 500MCS in the BSC. Close all clamps on the device except large filter line. Sterile weld the red harvest line from the G-Rex 500MCS to the pump tubing outlet line. Connect bioprocess bag female luer to male luer of the Pump boot. Hang the bioprocess bag on the IV pole, open the clamps and pump 4.5 Liters of the CM2 media into the G-Rex 500MCS. Clear the line, clamp, and heat seal. Place G-Rex 500MCS in the incubator.

[001776] Feeder Cells. Record the dry weight of a 1L transfer pack (TP). Pump 500mL CM2 by weight into the TP. Verify and Log out feeder cell bags. Thaw feeder cells in the 37° C (+/- 1° C) water bath. Record temperature of water bath. Dispense feeder cells into the TP using a syringe. Heat seal the TP and place in incubator. Perform a cell count on the feeder cell sample and calculate the total viable cell density in the TP.

[001777] Using a 1mL syringe and 18G needle draw up OKT3, transfer to the feeder TP through the female luer. Sterile weld the feeder bag to the red line on the G-Rex 500MCS. Unclamp the line and allow the feeder cells to flow into the flask by gravity.

[001778] Added tumor reactive TILs to G-REX500MCS. Heat seal. Incubate G-REX500MCS at 37.0 ± 2.0 °C, CO₂ Percentage: 5.0 ± 1.5 %CO₂.

[001779]

[001780] Day 5

[001781] Media Preparation. Warm one 10L bag of CM4 in a 37°C incubator at least 30 minutes or until ready to use. Remove the G-Rex 500MCS flask from the incubator and place on the benchtop adjacent the GatheRex. Check all clamps are closed except large filter line. Move the clamp on the quick connect line close to the “T” junction. Heat seal a 2L transfer pack 2” below the “Y removing the spike and record dry weight. Sterile weld the 2L TP to the clear collection line on the G-Rex 500MCS. After removal of the supernatant, swirl the flask until all the cells have been detached from the membrane. Release all clamps leading to the 2L TP and using the GatheRex transfer the residual cell suspension into the 2L TP.

[001782] Perform single cell counts and record data and attach counting raw data to batch record. Document Dilution. Document the Cellometer counting program. Verify the correct dilution is entered into the Cellometer.

[001783] Attach an 18G needle to a 10mL syringe and draw 5mL of IL-2 into the syringe (final concentration is 3000 IU/ml). Remove the needle and aseptically attach the syringe to the plasma transfer set and dispensed IL-2 into a 10L bag of Aim V with Glutamax.

[001784] Prepare G-REX500MCS Flasks. Determine amount of CM4 to add to flasks. Record volume of cells added per flask and volume of CM4 5000mL-A. Pump determined amount of CM4 into the G-Rex 500MCS using lines on flask as guide. Split the TILs from the G-REX500MCS and add to the flasks.

[001785] Monitor Incubator parameters: Temperature LED display: 37.0 ± 2.0 °C, CO₂ Percentage: 5.0 ± 1.5 %CO₂. Remove G-REX500MCS from the incubator. Prepare and label 1 L Transfer Pack as TIL Suspension and weigh 1L.

[001786] Day 11

[001787] Close all clamps on a 10 L Labtainerbag. Attach Baxter extension set to the 10L bag via luer connection. Sterile weld the red media removal line from the G-Rex 500MCS to the

extension set on the 10L bioprocess bag. Sterile weld the clear cell removal line from the G-Rex 500MCS to a 3L collection bag and label “pooled cell suspension”. Using the GatherRex pump, volume reduce the first flask.

[001788] Close the clamp on the supernatant bag and red line. Swirl the G-REX 500M flask until the TILs are completely resuspended while avoiding splashing or foaming. Make sure all cells have been dislodged from the membrane. Open clamps on clear line and 3L cell bag. Start GatherRex to collect the cell suspension. Close clamp on the line to the cell collection bag. Release clamps on GatherRex.

[001789] Repeat the above steps for each flask.

EXAMPLE 8: EXEMPLARY EMBODIMENT OF THE PROCESS FOR ENRICHING TUMOR REACTIVE TILS USING AUTOLOGOUS ORGANOID/TUMOROIDS

[001790] Fig. 3 illustrates an embodiment of the processes for enriching tumor reactive TILs using autologous organoids/tumoroids using the protocols of Example 6.

Tumor Preparation

[001791] A tumor sample freshly resected from a cancer patient is fragmented into approximately 2-6-mm³ fragments, and randomly distributed to provide material for: (1) production of pre-REP TIL product; and (2) cryopreservation of autologous tumor digest, both of which can proceed independently and simultaneously.

[001792] Tumor Processing. Obtain tumor specimen and transfer into suite at 2-8 °C immediately for processing. Aliquot tumor wash media. Tumor wash 1 is performed using 8” forceps (W3009771). The tumor is removed from the specimen bottle and transferred to the “Wash 1” dish prepared. This is followed by tumor wash 2 and tumor wash 3. Measure and assess tumor. Assess whether > 30% of entire tumor area observed to be necrotic and/or fatty tissue. Clean up dissection if applicable. If tumor is large and >30% of tissue exterior is observed to be necrotic/fatty, perform “clean up dissection” by removing necrotic/fatty tissue while preserving tumor inner structure using a combination of scalpel and/or forceps. Dissect tumor. Using a combination of scalpel and/or forceps, cut the tumor specimen into even, appropriately sized fragments (up to 6 intermediate fragments). Transfer intermediate tumor fragments. Dissect tumor fragments into pieces approximately 3x3x3mm in size. Store Intermediate Fragments to

prevent drying. Repeat intermediate fragment dissection. Determine number of pieces collected. If desirable tissue remains, select additional favorable tumor pieces from the “favorable intermediate fragments” 6-well plate to fill the drops for a maximum of 50 pieces.

Production of PreREP TIL Product

[001793] Day 0

[001794] CM1 Media Preparation. In a biological safety cabinet (BSC) add reagents to RPMI 1640 Media bottle. Add 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).

[001795] Remove unnecessary materials from BSC. Pass out media reagents from BSC, leave Gentamicin Sulfate and HBSS in BSC for Formulated Wash Media preparation.

[001796] Thaw IL-2 aliquot. Thaw one 1.1 mL IL-2 aliquot (6×10^6 IU/mL) (BR71424) until all ice has melted. Record IL-2: Lot # and Expiry.

[001797] 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. Add CM1 Day 0 Media 1 bottle and IL-2 (6×10^6 IU/mL) 1.0 mL.

[001798] Pass G-REX100MCS into BSC. Aseptically pass G-REX100MCS (W3013130) into the BSC.

[001799] Pump all Complete CM1 Day 0 Media into G-REX100MCS flask. Tissue Fragments Conical or GRex100MCS.

[001800] Tumor Wash Media Preparation. In the BSC, add 5.0 mL Gentamicin (W3009832 or W3012735) to 1 x 500 mL HBSS Media (W3013128) bottle. Add per bottle: HBSS (500.0 mL); Gentamicin sulfate, 50 mg/mL (5.0 mL). Filter HBSS containing gentamicin prepared through a 1L 0.22-micron filter unit (W1218810).

[001801] Prepared conical tube. Transfer tumor pieces to a 50 mL conical tube. Prepare BSC for G-REX100MCS. Remove G-REX100MCS from incubator. Aseptically pass G-REX100MCS flask into the BSC. Add tumor fragments to G-REX100MCS flask. Evenly distributed pieces.

- [001802] 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.
- [001803] The pre-REP step is performed by culturing ≤ 50 tumor fragments in a G-REX-100MCS flask in the presence of CM1 with 6000 IU/mL IL-2 for 6–9-days.
- [001804] After process is complete, discard any remaining warmed media and thawed aliquots of IL-2.
- [001805] TIL Harvest. Preprocessing table. Incubator parameters: Temperature LED display: 37.0 ± 2.0 °C; CO₂ Percentage: 5.0 ± 1.5 % CO₂. Remove G-REX100MCS from incubator. Prepare 300 mL Transfer Pack. Weld transfer packs to G-REX100MCS.
- [001806] Prepare flask for TIL Harvest and initiation of TIL Harvest. Using the GatheRex, transfer the cell suspension through the blood filter into the 300 mL transfer pack. Inspect membrane for adherent cells.
- [001807] Rinse flask membrane. Close clamps on G-REX100MCS. Ensure all clamps are closed. Heat seal the TIL and the “Supernatant” transfer pack. Calculate volume of TIL suspension. Prepare Supernatant Transfer Pack for Sampling.
- [001808] Incubate TIL. Place TIL transfer pack in incubator until needed. Perform cell counts and calculations. Determine the Average of Viable Cell Concentration and Viability of the cell counts performed. Viability $\div 2$. Viable Cell Concentration $\div 2$. Determine Upper and Lower Limit for counts. Lower Limit: Average of Viable Cell Concentration $\times 0.9$. Upper Limit: Average of Viable Cell Concentration $\times 1.1$. Confirm both counts within acceptable limits. Determine an average Viable Cell Concentration from all four counts performed.
- [001809] The procedures for obtaining cell and viability counts use the Nexcelom Cellometer K2 or equivalent cell counter.
- [001810] Adjust Volume of TIL Suspension: Calculate the adjusted volume of TIL suspension after removal of cell count samples. Total TIL Cell Volume (A). Volume of Cell Count Sample Removed (4.0 mL) (B) Adjusted Total TIL Cell Volume $C = A - B$.
- [001811] Calculate Total Viable TIL Cells. Average Viable Cell Concentration*: Total Volume; Total Viable Cells: $C = A \times B$.

Tumor Processing and Digestion

[001812] At the same time or after the initiation of the preREP process, start tumor processing and digestion.

[001813] If using GentleMACS OctoDissociator, transfer the tumor fragments to a GentleMACS C-Tube (C-tube) or 50-ml conical tube in the 5-ml of digest cocktail (in HBSS) indicated above. Transfer 2-3 fragments (4-6mm) to each C-tube.

[001814] Transfer each C-tube (Miltenyi Biotec, Germany, 130-096-334) to the GentleMACS OctoDissociator (Miltenyi Biotec, Germany, 130-095-937). Use according to the manufacturer's directions. Note, each tumor histology has a recommended program for tumor dissociation. Select the appropriate program for the respective tumor histology. The dissociation would approximately one hour.

[001815] If the GentleMACS OctoDissociator is not available, use a standard rotator. Placed 2-3 tumor fragments in a 50-ml conical tube (sealed with parafilm to avoid leakage) and secure to the rotator. Place the rotator, at 37°C, 5% CO₂ humidified incubator on constant rotation for 1-2 hours. Alternatively, the tumor fragments could be digested at RT overnight, also with constant rotation.

[001816] Post-digest, remove the C-tube from the Octodissociator or rotator. Attach a 0.22- μ m strainer to sterile Falcon conical tube. Using a pipette, pass all contents from the C-tube/ or 50-ml conical (5ml) through the 0.22- μ m strainer into a 50-ml conical. Wash the C-tube/50-ml conical with 10-ml of HBSS and apply to the strainer. Use the flat end of a sterile syringe plunger to dissociate any remaining non-digested tumor through the filter. Add CM1 or HBSS up to 50-ml and cap the tube.

[001817] Pellet the samples by centrifugation, 1500 rpm, 5 min at RT (with an acceleration and deceleration of 9). Carefully remove the liquid, resuspended pellet in 5-ml of CM1 for cell counting and viability assessment.

[001818] Put aside whole tumor digest for the following: 1. Cell culture (unselected TIL control) 2. FMO flow cytometry controls 3. Pre-sort whole tumor digest phenotyping assays 4. Frozen for tumor reactivity/cell killing assays. The number of cells put aside will depend on the total digest yield and tumor histology.

Enzyme Preparation for Tumor Digestion (Using Research Grade DNase, Collagenase and Hyaluronidase)

[001819] Reconstitute the lyophilized enzymes in the amount of sterile HBSS indicated for each of the digestion enzymes below. These enzymes are prepared as 10X. Pipette up and down several times and swirl to ensure complete reconstitution.

[001820] Reconstitute 1-g of Collagenase IV (Sigma, MO, C5138) in 10-ml HBSS (to make a 100-mg/mL stock). Mix by pipetting up and down to dissolve. If not dissolved after reconstitution, place in a 37°C H₂O bath for 5 minutes. Aliquot into 1-ml vials. This is the 100-mg/mL 10X working stock for collagenase.

[001821] Prepare the DNase (Sigma, MO, D5025) stock solution (10,000-IU/mL). The units of DNase for each lot is provided in the accompanying data sheet. Calculate the appropriate volume of HBSS to reconstitute the 100-mg lyophilized DNase stock. For example, if the DNase stock is 2000-U/mg, the total DNase in the stock is 200,000-IU (2000-IU/mg X 100-mg). Dilute to a working stock of 10,000IU, add 20-ml of HBSS to the 100mg of DNase (200,000IU/20ml=10,000U/mL). Aliquot into 1-ml vials. This is the 10,000IU/mL 10X working stock for DNase.

[001822] Prepare the hyaluronidase 10-mg/mL (Sigma, MO, H2126) stock solution. Reconstitute the 500-mg vial with 50-ml of HBSS to make a 10-mg/mL stock solution. Aliquot into 1-ml vials. This was the 10-mg/mL 10X working stock for hyaluronidase.

[001823] Dilute the stock digest enzymes to 1X. To make a 1X working solution, add 500-ml each of the collagenase, DNase and hyaluronidase to 3.5-ml of HBSS. Add the digest cocktail directly to the C-tube.

Enzyme Preparation for Tumor Digestion (using GMP Collagenase and Neutral Protease)

[001824] Reconstitute the lyophilized enzymes in the amount of sterile HBSS indicated for each of the digestion enzymes below. Be sure to capture any residual powder from the sides of the bottles and from the protective foil on the bottles opening. Pipette up and down several times and swirl to ensure complete reconstitution.

[001825] Reconstitute the Collagenase AF-1 (Nordmark, Sweden, N0003554) in 10-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 2892 PZ U/vial. After reconstitution the collagenase stock was 289.2 PZ U/mL.

[001826] Reconstitute the Neutral protease (Nordmark, Sweden, N0003553) in 1-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 175 DMC U/vial. Therefore, after reconstitution the neutral protease stock was 175 DMC/mL.

[001827] Reconstitute the DNase I (Roche, Switzerland, 03724751) in 1-ml of sterile HBSS. The lyophilized stock enzyme is at a concentration of 4KU/vial. Therefore, after reconstitution the DNase stock is 4KU/vial.

[001828] Prepare the working GMP digest cocktail. Add 10.2- μ l of the neutral protease (0.36 DMC U/mL), 21.3- μ l of collagenase AF-1 (1.2 PZ/mL) and 250- μ l of DNase I (200 U/mL) to 4.7-ml of sterile HBSS. Place the digest cocktail directly into the C-tube.

Cleaning up the Digest using the Debris Removal Kit

[001829] Debris could be removed from the tumor digest using the Debris Removal Solution (Miltenyi Biotec, Germany, Cat#130-109-398) or other equivalent reagent, according to the manufacturer's directions.

[001830] Centrifuge the tumor cell suspension at 300Xg for 10 minutes at 4°C and aspirate supernatant completely.

[001831] Resuspend cell suspension carefully with the appropriate volume of cold buffer according to the table below and transfer the cell suspension to a 15ml conical tube. DO NOT VORTEX.

	<u>Resuspension (PBS)</u>	<u>Debris Removal Solution</u>	<u>Overlay (PBS)</u>
0.5-1g tissue	6200- μ l	1800- μ l	4-ml
> 0.5 g tissue	3100- μ l	900- μ l	4-ml

[001832] Add appropriate volume of cold Debris Removal Solution and mix well by pipetting slowly up and down 10-20 times using a 5-ml pipette. Overlayed very gently with 4-ml of cold buffer. Tilt the tube and pipette very slowly to ensure that the PBS/D-PBS phase overlays the cell suspension and phases are not mixed. Centrifuge the tumor cell suspension at 3000Xg for

10 minutes at 4°C with full acceleration and full break. Three phases should form. Aspirate the two top phases completely and discard them.

[001833] The bottom phase contains the Debris Removal Solution and the cells. Be sure to leave at least as much volume at the bottom as was added of the Debris Removal solution. (i.e. if 1ml of solution was added leave at least 1-ml at the bottom of the tube).

[001834] Bring up to 15-ml with cold buffer and invert the tube at least three times. DO NOT VORTEX. Centrifuge at 4°C and 1000Xg for 10 minutes with full acceleration and full break. Resuspend cells in HBSS or media for cell count.

Co-Culture with Autologous Organoids/Tumoroids

[001835] Add the autologous organoids/tumoroids generated using the protocol of Example 10 to the pre-REP culture media at a ratio of 1:5 organoid/tumoroid:TIL and allow the co-culture of pre-REP'd TILs and the autologous organoids/tumoroids to proceed for 24-72 hours. In some embodiments, the residual tumor fragments will be removed from the pre-REP cell culture medium before co-culturing with the autologous organoids/tumoroids.

[001836] After the co-culture, the supernatant containing the cells is removed, and the wells are washed with media and the bottom of the flask/well is lightly scraped using a Teflon policeman to remove the organoids/tumoroids. The suspension containing the organoids/tumoroids is filtered, and disrupted over a 70-μ filter, to elute any endogenous or infiltrating TILs.

Staining Co-Cultured TILs for Cell Sorting

[001837] The co-cultured TILs are stained with a cocktail that includes anti-4-1BB, and anti-OX40 antibodies according to the following protocol. Post-co-culture, resuspend the TILs in 10-ml HBSS.

[001838] Resuspend pellet in FACS buffer (1 X HBSS, 1mM EDTA, 2% fetal bovine serum). The amount of FACS buffer added to the pellet is based upon the size of the pellet. The staining volume should be about 3 times the size of the pellet. Therefore, if there is 300- μl of cells, the volume of buffer should be at least 900-μl.

[001839] For antibody addition, each 100-μl of volume is equivalent to one test (titered amount of antibody). *I.e.*, if there is 1-ml of volume, 10X the amount of titered antibody is

required. Add a titrated amount of each of the following antibodies; anti-4-1BB-PE-Cy7, and anti-OX40 FITC per 100- μ l of volume. Incubate cells on ice for 30 minutes. Protect from light during incubation. Agitate a couple times during incubation. Resuspend cells in 20-ml of FACS buffer. Pass solution through a 70-micron cell strainer into a new 50-ml conical. Centrifuge, 400Xg, 5 min at RT (acceleration and deceleration of 9). Aspirate. Resuspend cells in up to 10e6/mL TOTAL (live+dead) in FACS buffer. Minimum volume is 300- μ l. Transfer to sterile polypropylene FACS tubes or 15-ml conical tubes. 3-ml/tube for FACS sorting. Prepare 15-ml collection tubes for the sorted populations. Place 2-ml of FACS buffer in the tubes.

Collection of Tumor Reactive TILs

[001840] To assess for reactivity of the TILs after the co-culture, 1.0 mL of the supernatant from the co-culture is assayed for IFN- γ level.

[001841] The co-cultured TILs are stained with anti-4-1BB and anti-OX40 antibodies for FACS sorting. After staining, the TILs are sorted using a SONY FX 500 cytometer by 2-color sorting. 4-1BB, OX40 double positive TILs are collected as a population of tumor reactive TILs.

REP of Tumor Reactive TILs

[001842] The collected tumor reactive TILs are further expanded using REP conditions for 11 days.

[001843] Day 0

[001844] Prepare CM2 and place at 4 °C.

[001845] Prepare G-Rex 500MCS Flask. Using 10 mL syringe aseptically transfer 0.5mL of IL-2 (stock is 6×10^6 IU/mL) for each liter of CM2 into the bioprocess bag through an unused sterile female luer connector. Open exterior packaging and place G-Rex 500MCS in the BSC. Close all clamps on the device except large filter line. Sterile weld the red harvest line from the G-Rex 500MCS to the pump tubing outlet line. Connect bioprocess bag female luer to male luer of the Pump boot. Hang the bioprocess bag on the IV pole, open the clamps and pump 4.5 Liters of the CM2 media into the G-Rex 500MCS. Clear the line, clamp, and heat seal. Place G-Rex 500MCS in the incubator.

[001846] Feeder Cells. Record the dry weight of a 1L transfer pack (TP). Pump 500mL CM2 by weight into the TP. Verify and Log out feeder cell bags. Thaw feeder cells in the 37° C (+/- 1° C) water bath. Record temperature of water bath. Dispense feeder cells into the TP using a syringe. Heat seal the TP and place in incubator. Perform a cell count on the feeder cell sample and calculate the total viable cell density in the TP.

[001847] Using a 1mL syringe and 18G needle draw up OKT3, transfer to the feeder TP through the female luer. Sterile weld the feeder bag to the red line on the G-Rex 500MCS. Unclamp the line and allow the feeder cells to flow into the flask by gravity.

[001848] Added tumor reactive TILs to G-REX500MCS. Heat seal. Incubate G-REX500MCS at 37.0±2.0 °C, CO₂ Percentage: 5.0±1.5 %CO₂.

[001849]

[001850] Day 5

[001851] Media Preparation. Warm one 10L bag of CM4 in a 37°C incubator at least 30 minutes or until ready to use. Remove the G-Rex 500MCS flask from the incubator and place on the benchtop adjacent the GatheRex. Check all clamps are closed except large filter line. Move the clamp on the quick connect line close to the “T” junction. Heat seal a 2L transfer pack 2” below the “Y removing the spike and record dry weight. Sterile weld the 2L TP to the clear collection line on the G-Rex 500MCS. After removal of the supernatant, swirl the flask until all the cells have been detached from the membrane. Release all clamps leading to the 2L TP and using the GatheRex transfer the residual cell suspension into the 2L TP.

[001852] Perform single cell counts and record data and attach counting raw data to batch record. Document Dilution. Document the Cellometer counting program. Verify the correct dilution is entered into the Cellometer.

[001853] Attach an 18G needle to a 10mL syringe and draw 5mL of IL-2 into the syringe (final concentration is 3000 IU/ml). Remove the needle and aseptically attach the syringe to the plasma transfer set and dispensed IL-2 into a 10L bag of Aim V with Glutamax.

[001854] Prepare G-REX500MCS Flasks. Determine amount of CM4 to add to flasks. Record volume of cells added per flask and volume of CM4 5000mL-A. Pump determined

amount of CM4 into the G-Rex 500MCS using lines on flask as guide. Split the TILs from the G-REX500MCS and add to the flasks.

[001855] Monitor Incubator parameters: Temperature LED display: 37.0 ± 2.0 °C, CO₂ Percentage: 5.0 ± 1.5 %CO₂. Remove G-REX500MCS from the incubator. Prepare and label 1 L Transfer Pack as TIL Suspension and weigh 1L.

[001856] Day 11

[001857] Close all clamps on a 10 L Labtainerbag. Attach Baxter extension set to the 10L bag via luer connection. Sterile weld the red media removal line from the G-Rex 500MCS to the extension set on the 10L bioprocess bag. Sterile weld the clear cell removal line from the G-Rex 500MCS to a 3L collection bag and label “pooled cell suspension”. Using the GatherRex pump, volume reduce the first flask.

[001858] Close the clamp on the supernatant bag and red line. Swirl the G-REX 500M flask until the TILs are completely resuspended while avoiding splashing or foaming. Make sure all cells have been dislodged from the membrane. Open clamps on clear line and 3L cell bag. Start GatherRex to collect the cell suspension. Close clamp on the line to the cell collection bag. Release clamps on GatherRex.

[001859] Repeat the above steps for each flask.

EXAMPLE 9: TUMOR-REACTIVE CELLS EXIST AT VARIOUS AMOUNTS IN TIL CULTURES

[001860] To test the feasibility of the TIL:tumor cell co-culture, 25 TIL lots produced using the Gen 2 process, produced from five melanoma, 16 NSCLC, and four HNSCC tumor samples, were thawed prior to co-culture with autologous tumor digests. Autologous tumor digests were processed using a dead cell removal kit (Miltenyi, CA) and co-cultured with the TILs at a 3:1 TIL:tumor cell ratio for 24 hours. Saturating concentration of anti-HLA class I antibody was used to demonstrate specificity. TIL alone +/- anti-HLA class I were included as negative controls. Levels of IFN- γ were detected using the ELLA assay. Results are plotted for individual samples (Fig. 4), with the black open dots representing the TIL + autologous tumor digest co-culture and the black open squares representing the TIL+ autologous tumor digest + anti-HLA samples. IFN- γ represents the level of secreted IFN- γ released from the TIL upon engagement

of the TCR to the HLA—peptide complex of the target line, above background levels (TIL alone +/- anti-HLA class I antibody). Black dotted line represents a baseline of 0. Of the 25 TIL evaluable tumor-reactivity co-cultures, 20 TIL lots produced greater than 100 pg/ml Δ IFN- γ (range 0 to 4341).

[001861] These results are proof that co-culture with autologous tumor digest is capable of inducing a strong, measurable response in at least the tumor-reactive subpopulation of a given TIL culture. Indeed, the wide variability observed proves the necessity of a process for enrichment, selection and expansion of these most tumor-reactive TILs for more robust therapeutics.

EXAMPLE 10: EXEMPLARY ORGANOID/TUMOROID CULTURE PROCEDURE

[001862] This example demonstrates one aspect of some embodiments of the processes for generating an organoid/tumoroid. All specific amounts, products, and steps in the following procedure are merely illustrative and are in no way limiting on any embodiments herein described. Further disclosure of these methods is described in Drost, J., *et al.* (2016). *Nature Protocols*, 11(2), 347-358, the content of which is herein incorporated by reference in its entirety.

PROCEDURE

Generation of tumor cells:

[001863] Prewarm tissue culture 24 well plate overnight at 37° C. On the day of the tumor arrival: Thaw grow matrix (based on histology, Cultrex RGF Basement Membrane Extract R&D, Cat#3433-010-01) on ice ~2 hours before starting this process. A scalpel will be used to mince tumor tissue into small pieces (~1–3 mm³). (A few 3 mm³ fragments will be reserved for fixation on day 0 for tissue staining comparison. Also, generate TIL from fragments based on MTH-0005).

[001864] The rest of the 1-3 mm³ fragments will be subjected to enzymatic digest using 1 ml of 5 mg/ml collagenase type II (Fisher Scientific, Cat#17101015) with 10 μ M Y-27632 dihydrochloride per ~50mg of minced tissue. Digestion will be done for 1 hour at the GentleMACS Dissociator (Miltenyi Biotec Inc., Germany, Cat#130-093-235).

[001865] The cells from undigested sections will be strained using a cell strainer (cell strainer 0.7 mm). Cells will be washed by topping up to 20ml with adDMEM/F12+++ (Fisher Scientific Cat#12634010) with 10 μ M Y-27632 dihydrochloride (R&D Cat#1254\1). Tubes will be centrifuged at 400g for 5 minutes at RT. Wash steps will be repeated as needed. Pellets will be resuspended the pellet in 1ml of adDMEM/F12+++ with 10 μ M Y-27632 dihydrochloride. Cells will be counted, for example using a using K2 cell counter.

Establishing organoids cultures

[001866] Will plate ~50,000 cells in a 40 μ L drop of gel in the middle of one well of a 24-well plate. Average final percentage of grow matrix should be ~75%. Plates will be placed upside down into the CO₂ incubator (5% CO₂, 37° C) for 20-30 minutes to allow the matrix gel to solidify. 400 μ l of prewarmed organoids culture media (based on histology; see Tables 39-42, included below) will be gently pipetted and will be placed into the CO₂ incubator (5% CO₂, 37°C).

adDMEM/F12+++ Medium Recipe

[001867] adDMEM/F12 (Fisher scientific, Cat #: 12634010) containing penicillin/streptomycin, 10 mM HEPES and 2 mM GlutaMAX (100x diluted).

Table 39: Breast Cancer Organoid Medium Recipe

Medium component	Supplier	Catalogue number	Final concentration
R-Spondin 1 conditioned medium	home made		10%
or R-Spondin 1	Peprtech	120-38	250 ng•ml ⁻¹
Neuregulin 1	Peprtech	100-03	5 nM
FGF 7	Peprtech	100-19	5 ng•ml ⁻¹
FGF 10	Peprtech	100-26	20 ng•ml ⁻¹
EGF	Peprtech	AF-100-15	5 ng•ml ⁻¹ ^a
Noggin	Peprtech	120-10C	100 ng•ml ⁻¹
A83-01	Tocris	2939	500 nM

Y-27632	Abmole	Y-27632	5 mM
SB202190	Sigma	S7067	500 nM ^b
B27 supplement	Gibco	17504-44	1x
N-Acetylcysteine	Sigma	A9165-5g	1.25 mM
Nicotinamide	Sigma	N0636	5 mM
GlutaMax 100x	Invitrogen	12634-034	1x
Hepes	Invitrogen	15630-056	10 mM
Penicillin/Streptomycin	Invitrogen	15140-122	100 U•ml ⁻¹ / 100 mg•ml ⁻¹
Primocin	Invivogen	Ant-pm-1	50 mg•ml ⁻¹
Advanced DMEM/F12	Invitrogen	12634-034	1x

a reduce/remove in case of sinking organoids,

b concentrations above 1 μM decrease organoid formation efficiency

Table 40: Lung Cancer Organoid Medium Recipe

Media component	Supplier	Catalogue number	Final concentration
R-Spondin 1	Peprtech	120-38	500 ng•ml ⁻¹
FGF 7	Peprtech	100-19	25 ng•ml ⁻¹
FGF 10	Peprtech	100-26	100 ng•ml ⁻¹
Noggin	Peprtech	120-10C	100 ng•ml ⁻¹
A83-01	Tocris	2939	500 nM
Y-27632	Abmole	Y-27632	5 mM
SB202190	Sigma	S7067	500 nMt
B27 supplement	Gibco	17504-44	1x
N-Acetylcysteine	Sigma	A9165-5g	1.25 mM
Nicotinamide	Sigma	N0636	5 mM
GlutaMax 100x	Invitrogen	12634-034	1x
Hepes	Invitrogen	15630-056	10 mM
Penicillin / Streptomycin	Invitrogen	15140-122	100 U•ml ⁻¹ / 100 mg•ml ⁻¹
Primocin	Invivogen	Ant-pm-1	50 mg•ml ⁻¹

Advanced DMEM/F12	Invitrogen	12634-034	1x
-------------------	------------	-----------	----

[001868] Lung Cancer Organoid Medium Recipe-Jang Lab. The medium comprises: DMEM/F12, 20ng/ml IGF2, 50ng/ml IEGF, N2 1x, B27 1x, 10uM Y27632, and 1% Pen-strep

Table 41: Prostate Cancer Organoid Medium Recipe

Factor	Mouse organoids	Human organoids
827	50x diluted	50x diluted
N-acetylcysteine	1.25 mM	1.25 mM
EGF	50 ng/ml	5 ng/ml
Noggin	100 ng/ml	100 ng/ml
R-spondin 1	500 ng/ml or 10% conditioned medium	500 ng/ml or 10% conditioned medium
A83-01	200 nM	500 nM
FGF10	—	10 ng/ml
FGF2	—	5 ng/ml
Prostaglandin E2	—	1 μ M
Nicotinamide	—	10 mM
513202190	—	10 μ M
DHT	1 nM	1 μ M
Y-2702 dihydrachtoride ^a	10 μ M	10 μ M

^a Y-2702 dihydrachtoride is only added to the medium during establishment of the culture and after passaging the organoids using Trypl I

Table 42: Colon Cancer Organoid Medium Recipe

Medium component	Supplier	Catalogue number	Final concentration	
------------------	----------	------------------	---------------------	--

R-Spondin 1 conditioned medium	home made			20%
or R-Spondin 1	R&D	3500-RS/CF	250 ng•ml ⁻¹	
PGE2	Peprtech		10 nM	
EGF	Peprtech	AF-100-15	50 ng•ml ⁻¹	
Noggin	Peprtech	120-10C	100 ng•ml ⁻¹	10%
A83-01	Tocris	2939	500 nM	
Y-27632	Abmole	Y-27632	5 mM	
SB202190	Sigma	S7067	10uM	3uM
B27 supplement	Gibco	17504-44	1x	
N-Acetylcysteine	Sigma	A9165-5g	1.25 mM	
Nicotinamide	Sigma	N0636	10 mM	
GlutaMAX		12634-034	2mM	Utaglutaminl/Lonza
Hepes	Invitrogen	15630-056	10 mM	Gibco
Penicillin/Streptomycin	Invitrogen	15140-122	100 U•ml ⁻¹ / 100 mg•ml ⁻¹	Gibco
Advanced DMEM/F12	Invitrogen	12634-034	1x	
Gastrin	Sigma-Aldrich Inc.	G9020-250UG	10nM	

H&N Cancer Organoid Medium Recipe

[001869] Organoid medium contained 1 x B27 supplement (Life Technologies, catalog no. 17504-044), 1.25 mmol/L N-acetyl-L-cysteine (Sigma-Aldrich, catalog no. A9165), 10 mmol/L Nicotinamide (Sigma-Aldrich, catalog no. N0636), 50 ng/nnl_ human EGF (PeproTech, catalog no. AF-100-15), 500 nmol/L A83-01, 10 ng/nnl_ human FGF10 (PeproTech, catalog no. 100-26), 5 ng/mL human FGF2 (PeproTech, catalog no. 100-18B), 1 µcool/L Prostaglandin E2 (Tocris Bioscience, catalog no. 2296), 0.3 µmol/L CHIR 99021 (Sigma-Aldrich, catalog no. SML1046), 1 µcool/L Forskolin [Bio-Techne (R&D Systems) catalog no. 1099], 4% R-spondin, and 4% Noggin (both produced via the r-PEX protein expression platform at U-Protein Express BV). Mouse organoids were maintained similar to human organoids, but were grown in +/+/, containing B27, 25 mnnol/L N-acetyl-L-cysteine, 10 mmol/L Nicotinamide, 2% RSPO, 50

ng/mL EGF, and 10 ng/mL FGF10. Organoids were split between 7 and 14 days after initial plating.

[001870] Media will be replaced every 2-3 days for 7 days. After 7 days, replace organoids culture media with media that does not contain Y-27632 dihydrochloride. Organoids will be passaged after 1-2 weeks, depending on size and density.

Subculture and cryopreservation of organoids

[001871] Media will be aspirated from the well and 1 mL of TrypLE (Fisher, Cat#12604013) containing 10 μ M Y-27632 dihydrochloride per well of matrix gel containing organoids will be added. Will pipette to break the gel and place it in CO₂ incubator (5% CO₂, 37° C) for 5-15 minutes. Organoids will be collected into 15ml sterile tube. The TrypLE will be inactivated by adding ~5ml of addMEM/F12+++ containing 5% FBS. Tubes will be centrifuged at 400g for 5 minutes at 4° C. If cryopreservation is desired, pellet will be resuspended with 0.5 mL CS-10 (per well of organoids). Cryopreserved vials can be stored at -80° C up to one month. For longer periods, vials should be stored in the liquid nitrogen tank.

To subculture:

[001872] The pellet will be resuspended in the desired amount of freshly thawed matrix gel. For example, for 1:2 ratio, resuspend in 80 μ L of matrix gel. Organoids should be split based on confluency and can be split between 1:2 to 1:6. 40 μ L drops will be plated into the middle of each 24-well plate. The dish will be placed into CO₂ incubator (5% CO₂, 37° C) for ~25-30 minutes to allow the matrix gel solidification. 400 μ L of prewarmed organoids culture media (based on histology) will be gently pipetted and the plate will be placed into the CO₂ incubator (5% CO₂, 37° C).

[001873] Media will be aspirated from the well. Well will be rinsed with PBS 1X. A 2% agarose gel will be prepared and allowed to cool but do not allow it to solidify. Approximately 1 mL of 2% agarose gel will be poured into the well containing the organoids matrix gel. The 2% agarose gel will be allowed to solidify. The gel will be removed from the 24 well carefully as it will contain the matrix gel. The gel will be cut to get rid of unnecessary gel sections. Verify the presence of the organoids in the gel. The organoids containing gel will be placed in 10%

PFA/Formalin solution (Fisher Scientific, Cat # 22026439) for overnight at 4° C. The gel will be moved to PBS 1X. The gel will be sent for paraffin embedding and staining (H&E/IHC).

[001874] The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the compositions, systems and methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Modifications of the above-described modes for carrying out the invention that are obvious to persons of skill in the art are intended to be within the scope of the following claims. All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains.

[001875] All headings and section designations are used for clarity and reference purposes only and are not to be considered limiting in any way. For example, those of skill in the art will appreciate the usefulness of combining various aspects from different headings and sections as appropriate according to the spirit and scope of the invention described herein.

[001876] All references cited herein are hereby incorporated by reference herein in their entireties and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

[001877] Many modifications and variations of this application can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. The specific embodiments and examples described herein are offered by way of example only, and the application is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which the claims are entitled.

WHAT IS CLAIMED IS:

1. A method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising:
 - (a) obtaining a tumor sample from a patient;
 - (b) dividing the tumor sample into a first portion and a second portion;
 - (c) performing a first expansion of a first population of TILs in the second portion of the tumor sample by culturing the second portion of the tumor sample in a first cell culture medium and IL-2 to produce a second population of TILs; and
 - (d) contacting the second population of TILs with tumor cells or tumor cell antigens from or derived from a tumor digest obtained by digesting the first portion of the tumor sample to generate a third population of TILs,wherein the third population of TILs comprises a plurality of tumor reactive TILs that is enriched in comparison to the second population of TILs.
2. The method of claim 1, wherein step (d) is performed for about 1 to about 3 days.
3. A method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising:
 - (a) obtaining a tumor sample from a patient;
 - (b) dividing the tumor sample into a first portion and a second portion;
 - (c) performing a first expansion of a first population of TILs in the second portion of the tumor sample by culturing the second portion of the tumor sample in a first cell culture medium and IL-2 to produce a second population of TILs; and
 - (d) contacting the second population of TILs with a population of mature dendritic cells (DCs) generated from culturing a population of immature DCs with tumor cells or tumor cell antigens from or derived from a tumor digest obtained by digesting the first portion of the tumor sample to generate a third population of TILs, wherein the third population of TILs comprises a plurality of tumor reactive TILs that is enriched in comparison to the second population of TILs.

4. The method of claim 3, wherein the population of immature DCs is generated by culturing a population of monocytes in the presence of GM-CSF and IL-4.
5. The method of claim 4, wherein the population of monocytes is obtained from PBMCs.
6. The method of claim 5, wherein the PBMCs are obtained from the patient.
7. The method of any one of claims 4-6, wherein the population of monocytes is cultured in the presence of GM-CSF and IL-4 for about 6 days.
8. The method of any one of claims 3-7, wherein culturing the population of immature DCs in the presence of tumor cells or tumor cell antigens from or derived from the tumor digest comprises generating tumor lysate from the tumor digest and culturing the immature DCs in the presence of tumor cells or tumor cell antigens from or derived from the tumor lysate.
9. The method of any one of claims 3-8, wherein the population of immature DCs is cultured with the tumor cells of the tumor digest at a ratio of about 3:1.
10. The method of any one of claims 3-9, wherein the population of immature DCs is cultured in the presence of tumor cells or tumor cell antigens from or derived from the tumor digest for about 24 hours.
11. The method of any one of claims 3-10, wherein the tumor digest or tumor lysate is subjected to dead cell removal prior to being cultured with the population of immature DCs.
12. The method of any one of claims 3-11, wherein culturing the population of immature DCs in the presence of tumor cells or tumor cell antigens from or derived from the tumor digest is performed in the presence of TNF α , IL-6 and IL-1 β .

13. The method of claim 12, wherein the concentration of TNF α is about 2000IU/ml.
14. The method of claims 12 or 13, wherein the concentration of IL-6 is about 2000IU/ml.
15. The method of any one of claims 12-14, wherein the concentration of IL-1 β is about 400IU/ml.
16. The method of any one of claims 3-15, wherein the second population of TILs are cultured with the mature DCs.
17. The method of any one of claims 3-16, wherein step (d) is performed for about 1-3 days.
18. A method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising:
- (a) obtaining a tumor sample from a patient;
 - (b) dividing the tumor sample into a first portion and a second portion;
 - (c) performing a first expansion of a first population of TILs in the second portion of the tumor sample by culturing the second portion of the tumor sample in a first cell culture medium and IL-2 to produce a second population of TILs; and
 - (d) contacting the second population of TILs with organoids generated from the first portion of the tumor sample to generate a third population of TILs, wherein the third population of TILs comprises a plurality of tumor reactive TILs that is enriched in comparison to the second population of TILs.
19. The method of claim 18, wherein generating organoids from the first portion of the tumor sample comprises digesting the first portion of the tumor sample to obtain a tumor digest and generating organoids from the tumor digest.
20. The method of claim 18 or 19, wherein generating organoids comprises:

- (a) driving the first portion of the tumor sample and an unpolymerized fluid matrix material through one or more channels of a microfluidics apparatus,
 - i. wherein the microfluidics apparatus controls the pressure, flow rate or pressure and flow rate within the one or more channels and maintains a temperature of 20 degrees C or less, so that tumor-derived cells or multiple tumor fragments in the tumor sample and the unpolymerized fluid matrix travel through the one or more channels in laminar flow,
 - (b) combining tumor-derived cells or multiple tumor fragments and the unpolymerized fluid matrix material within the microfluidics apparatus to form a plurality of droplets of unpolymerized mixture, and
 - (c) exposing the plurality of droplets of unpolymerized mixture to a temperature of greater than 25 degrees C to polymerize the fluid matrix material and form the organoid.
21. The method of any one of claims 1-20, further comprising identifying the plurality of tumor reactive TILs in the third population of TILs.
22. The method of claim 21, wherein identifying the plurality of tumor reactive TILs comprises determining if a TIL exhibits an activation signal identifying the TIL as tumor reactive.
23. The method of claim 22, wherein the activation signal comprises increased and/or decreased cell surface expression of one or more proteins.
24. The method of claim 23, wherein the one or more proteins are selected from the group consisting of: CD3, CD4, CD8, PD-1, LAG3, Tim3, TIGIT, CD103, CD39, CD134, CD137, CD25, CD69, HLA-DR, CD107a, CD40L, Ki46, CD45RA, CCR7, and KLRG1.
25. The method of claim 23 or 24, wherein the cell surface expression of the one or more proteins is determined by flow cytometry.

26. The method of claim 25, wherein the flow cytometry is performed using a SONY FX 500, Miltenyi Tyto or Miltenyi CliniMACS flow-activated cell sorter.
27. The method of claim 22, wherein the activation signal comprises a cell morphology.
28. The method of claim 27, wherein the cell morphology is a flattened, rounded cell morphology.
29. The method of claim 22, wherein the activation signal is a concentration of mitochondrial mass in proximity to the cell membrane of the TIL.
30. The method of any one of claims 27-29, wherein the activation signal is determined by an imaging-based cell separation method.
31. The method of any one of claims 21-30, further comprising collecting the identified plurality of tumor reactive TILs.
32. The method of claim 31, wherein collecting the plurality of tumor reactive TILs comprises separating the plurality of tumor reactive TILs from non-tumor reactive TILs in the third population of TILs.
33. The method of claim 32, wherein the separating the plurality of tumor reactive TILs comprises removing non-tumor reactive TILs from the third population of TILs.
34. The method of any one of claims 1-33, wherein the method further comprises performing the step of:
 - (e) performing a second expansion by culturing the third population of TILs or the collected plurality of tumor reactive TILs in a second cell culture medium supplemented with additional IL-2, OKT-3 and antigen-presenting cells to generate a fourth population of TILs.

35. The method of any one of claims 1-34, wherein the first portion of the tumor sample comprises approximately one-third of the tumor sample.
36. The method of any one of claims 1-34, wherein the second portion of the tumor sample comprises approximately one half of the tumor sample.
37. The method of any one of claims 1-34, wherein the second portion of the tumor sample comprises approximately one third of the tumor sample.
38. The method of any one of claims 1-17 and/or 19-37, wherein the tumor digest undergoes 1, 2, 3, 4, 5, or 10 freeze-thaw cycles.
39. The method of any one of claims 1-38, wherein the first portion of the tumor sample comprises at least three million cells.
40. The method of any one of claims 34-39, wherein steps (a) through (e) are performed within a period of about 17 days to about 24 days, within a period of about 18 days to about 22 days, within a period of about 20 days to about 22 days, or within a period of about 22 days.
41. The method of any one of claims 1-40, wherein the first cell culture medium further comprises a factor selected from the group consisting of: IL-7, IL-15, IL-21, IL-12, Leukemia Inhibitory Factor (LIF), beta fibroblast growth factor (bFGF), and combinations thereof.
42. The method of any one of claims 1-41, wherein the second cell culture medium further comprises a factor selected from the group consisting of: IL-7, IL-15, IL-21, IL-12, LIF, bFGF, 41BBL, OX40L, CD86, CD64, and combinations thereof.
43. The method of any one of claims 1-42, wherein the tumor sample is selected from the group consisting of a solid tumor, a fine needle aspirate, and a small biopsy.

44. The method of any one of claims 22-43, wherein the activation signal comprises an increase in secreted interferon gamma (IFN γ).
45. The method of any one of claims 1-44, wherein the cell culture medium is provided in a container selected from the group consisting of a G-container and a Xuri cellbag.
46. The method of any one of claims 1-45, further comprising gene-editing the second population of TILs, the third population of TILs, or the plurality of tumor reactive TILs.
47. A pharmaceutical composition for the treatment of cancer comprising a population of TILs generated using the methods of any one of claims 1-46 and/or the fourth population of TILs generated using the methods of any one of claims 34-46.
48. The composition of claim 47, wherein the cancer is selected for the group consisting of melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, triple negative 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.
49. The composition of claim 47 or 48, further comprising a cryopreservant.
50. The composition of claim 49, wherein the cryopreservant comprises dimethylsulfoxide.
51. The composition of claim 47 or 48, further comprising a cryopreservant and an isotonic agent.
52. The composition of claim 51, further comprising a cryopreservant comprising dimethylsulfoxide and an isotonic agent comprising sodium chloride, sodium gluconate, and sodium acetate.

53. The composition of claim 52, further comprising a cryopreservant comprising dimethylsulfoxide and dextran 40 and an isotonic agent comprising sodium chloride, sodium gluconate, and sodium acetate.
54. The composition of any one of claims 47-53, wherein the composition is provided in a sterile infusion bag.
55. The composition of any one of claims 47-54, wherein said composition is harvested using a LOVO cell processing system.
56. The composition according to any of claims 47-55, wherein the TILs are gene-edited.