

## CYTOKINE ENCODING LENTIVIRAL VECTORS AND USES THEREOF FOR MAKING TUMOR INFILTRATING LYMPHOCYTES

### CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/513,550, filed on July 13, 2023, U.S. Provisional Patent Application No. 63/622,008, filed on January 17, 2024, U.S. Provisional Patent Application No. 63/562,210, filed on March 6, 2024, and U.S. Provisional Patent Application No. 63/649,302, filed on May 17, 2024, which are hereby incorporated by reference in their entireties.

### SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing, which has been submitted electronically in XML ST.26 format and is hereby incorporated by reference in its entirety. Said XML copy, created on July 10, 2024, is named 116983-5126-WO\_Sequence\_Listing and is 209,365 bytes in size.

### BACKGROUND

[0003] TIL cell therapy has shown clinical benefit for patients with solid tumors (Chesney et al., *Journal for ImmunoTherapy of Cancer* **2022**;10:e005755; 2. Schoenfeld et al., *SITC Annual Meeting* **2021**). However, an immunosuppressive tumor microenvironment (TME) may abrogate the full potential of TIL cell therapy (Granhøj et al., *Expert Opin Biol Th.* **2022**;1–15). The proinflammatory cytokine IL-12, known for its capability to increase IFN- $\gamma$  production and promote type 1 immune responses, reshapes the TME and has a potential to augment antitumor activity.

[0004] The current TIL expansion process results in both tumor specific TILs (TS-TILs) and bystander TILs in the expanded TIL product. The frequency of neoantigen-specific clones in TIL infusion products, and their ability to mobilize upon *in vitro* expansion correlate with TIL efficacy in melanoma (Kristensen N.P., et al. *J Clin. Invest.* **2022**;132:e150535; Chiffelle, J., et al., *bioRxiv* **2023**; the contents of which are hereby incorporated by reference in their entireties). Bystander T cells can have a Treg-like effect of stealing IL-2 and homostatic cytokines from effector TS-TILs. Bystander TILs also outcompete TS-TIL for nutrients and oxygen during the REP period, reducing their total numbers because: a) bystander TILs tend to be younger and fitter (less-differentiated) T cells and thus have more

proliferative potential; and b) TIL growth is dependent on metabolic state and phenotypic state of TILs, i.e. expression of costimulatory receptors. Bystander TILs take up valuable tumor real-estate after adoptive transfer and create a “traffic jam” getting to tumor. Allen, *et al.*, *Science*, **2022**, 378, eaba1624.

[0005] Thus, there remains a need for improved TIL therapies for the treatment of cancers. Disclosed herein are gene-edited TILs with an inducible and membrane-bound IL-12, which showed superior cytotoxic function. The present invention further provides an improved expansion process for preferentially producing TS-TILs over bystander TILs.

#### BRIEF SUMMARY

[0006] Some embodiments of the present disclosure provide a nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter.

[0007] Some embodiments of the present disclosure provide a nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter, and wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.

[0008] Some embodiments of the present disclosure provide a nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter, and a nucleotide sequence encoding a tethered IL-15 (TeIL-15), wherein the TeIL-15 is under the control of an EF1 $\alpha$  promoter.

[0009] Some embodiments of the present disclosure provide a nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter, and a nucleotide sequence encoding a tethered IL-15 (TeIL-15), wherein the TeIL-15 is under the control of an EF1 $\alpha$  promoter, and wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.

[0010] In some embodiments, the TeIL-12 comprises a membrane anchor, and a human IL-12 p40 subunit fused to a human IL-12 p35 subunit. In some embodiments, the human IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60 and the human IL-12 p40 subunit has the amino acid sequence of SEQ ID NO:61. In some embodiments, the TeIL-12



comprises an amino acid sequence set forth in SEQ ID NO:62. In some embodiments, the nucleotide sequence encoding the TeIL-12 is set forth in SEQ ID NO:162. In some embodiments, the nucleic acid molecule further comprises a nucleotide sequence encoding a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNF $\alpha$ , IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof. In some embodiments, the cytokine is under the control of a constitutive promoter. In some embodiments, the constitutive promoter is selected from the group consisting of an EF1 $\alpha$  promoter, a CMV promoter, a CAG promoter, an MND promoter, and an SSFV promoter. In some embodiments, the cytokine is under the control of a constitutive promoter. In some embodiments, the constitutive promoter is an EF1 $\alpha$  promoter. In some embodiments, the cytokine is IL-15 or a variant thereof. In some embodiments, the IL-15 is a human IL-15. In some embodiments, the human IL-15 has the amino acid sequence of SEQ ID NO:64. In some embodiments, the IL-15 is a tethered IL-15 (TeIL-15). In some embodiments, the TeIL-15 comprises a membrane anchor, and a human IL-15. In some embodiments, the TeIL-15 has the amino acid sequence of SEQ ID NO:73. In some embodiments, the cytokine is IL-2 or a variant thereof. In some embodiments, the IL-2 is a human IL-2. In some embodiments, the human IL-2 has the amino acid sequence of SEQ ID NO:138. In some embodiments, the IL-2 is a tethered IL-2 (TeIL-2). In some embodiments, the TeIL-2 has the amino acid sequence of SEQ ID NO:139. In some embodiments, the nucleic acid molecule further comprises a nucleotide sequence encoding a truncated CD19 (tCD19). In some embodiments, the nucleic acid molecule comprises the nucleic acid sequence set forth in SEQ ID NO:148-150. In some embodiments, the nucleic acid molecule further comprises a nucleotide sequence encoding an shRNA. In some embodiments, the shRNA inhibits the expression of an immune checkpoint gene. In some embodiments, the immune checkpoint gene is selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the shRNA is a PD-1 shRNA. In some embodiments, the PD-1 shRNA comprises a nucleic acid sequence set forth in SEQ ID NO:141-147. In some embodiments, the nucleic acid molecule comprises the nucleic acid sequence set forth in SEQ ID NO:151.

[0011] Some embodiments of the present disclosure provide a recombinant expression vector comprising the nucleic acid molecule disclosed herein.

[0012] Some embodiments of the present disclosure provide a recombinant expression vector comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter.

[0013] Some embodiments of the present disclosure provide a recombinant expression vector comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter, and wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.

[0014] Some embodiments of the present disclosure provide a recombinant expression vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter, and a nucleotide sequence encoding a tethered IL-15 (TeIL-15), wherein the TeIL-15 is under the control of an EF1 $\alpha$  promoter.

[0015] Some embodiments of the present disclosure provide a recombinant expression vector comprising a nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter, and a nucleotide sequence encoding a tethered IL-15 (TeIL-15), wherein the TeIL-15 is under the control of an EF1 $\alpha$  promoter, and wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.

[0016] Some embodiments of the present disclosure provide a recombinant expression vector comprising a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:166.

[0017] In some embodiments, the vector is a transfer vector. In some embodiments, the transfer vector is derived from the human immunodeficiency virus-1 (HIV-1) and is replication deficient. In some embodiments, the transfer vector is a pLenti-IRES-EGFP vector.

[0018] Some embodiments of the present disclosure provide a lentiviral expression system comprising the recombinant expression vector disclosed herein and one or more helper plasmids encoding an Env protein, a Gag protein, a Pol protein, and a Rev protein. In some

embodiments, the lentiviral expression system comprises a helper plasmid encoding the Env protein, the Gag protein, the Pol protein, and the Rev protein. In some embodiments, the lentiviral expression system comprises a helper plasmid encoding the Env protein, and a helper plasmid encoding the Gag protein, the Pol protein, and the Rev protein. In some embodiments, the lentiviral expression system comprises a helper plasmid encoding the Env protein, the Gag protein, and the Pol protein, and a helper plasmid encoding the Rev protein. In some embodiments, the lentiviral expression system comprises a helper plasmid encoding the Env protein and the Rev protein, and a helper plasmid encoding the Gag protein and the Pol protein. In some embodiments, the Env protein is selected from the group consisting of Ba-EVTR, VSV-G, and RD114. In some embodiments, the Env protein comprises a Ba-EVTR protein.

**[0019]** Some embodiments of the present disclosure provide a method of making a recombinant lentiviral particle, comprising: a) culturing a population of packaging cells in a cell culture medium; b) contacting the population of packaging cells with a lentiviral expression system disclosed herein; and c) harvesting the supernatant of the cell culture medium, wherein the supernatant comprises the recombinant lentiviral particle.

**[0020]** Some embodiments of the present disclosure provide a recombinant lentiviral RNA molecule comprising a nucleic acid molecule disclosed herein.

**[0021]** Some embodiments of the present disclosure provide a recombinant lentiviral proviral DNA molecule comprising a nucleic acid molecule disclosed herein.

**[0022]** Some embodiments of the present disclosure provide a recombinant lentiviral particle comprising a recombinant lentiviral RNA molecule disclosed herein. In some embodiments, the recombinant lentiviral particle is produced by a packaging cell line disclosed herein. In some embodiments, the recombinant lentiviral particle comprises an Env protein selected from the group consisting of Ba-EVTR, VSV-G, and RD114. In some embodiments, the recombinant lentiviral particle comprises an Env protein that is Ba-EVTR. In some embodiments, the recombinant lentiviral particle further comprises a reverse transcriptase. In some embodiments, the recombinant lentiviral particle further comprises a capsid enclosing the recombinant lentiviral RNA molecule.

**[0023]** Some embodiments of the present disclosure provide a gene-edited tumor infiltrating lymphocyte (TIL) comprising a recombinant lentiviral proviral DNA disclosed

herein. In some embodiments, the recombinant lentiviral proviral DNA is integrated into the genome of the gene-edited TIL.

**[0024]** Some embodiments of the present disclosure provide a gene-edited tumor infiltrating lymphocyte (TIL) expressing an exogenous tethered IL-12 (TeIL-12).

**[0025]** Some embodiments of the present disclosure provide a gene-edited tumor infiltrating lymphocyte (TIL) expressing an exogenous tethered IL-12 (TeIL-12), wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.

**[0026]** Some embodiments of the present disclosure provide a gene-edited tumor infiltrating lymphocyte (TIL) expressing an exogenous tethered IL-12 (TeIL-12), and an exogenous tethered IL-15 (TeIL-15).

**[0027]** Some embodiments of the present disclosure provide a gene-edited tumor infiltrating lymphocyte (TIL) expressing an exogenous tethered IL-12 (TeIL-12), and an exogenous tethered IL-15 (TeIL-15), wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.

**[0028]** Some embodiments of the present disclosure provide a gene-edited tumor infiltrating lymphocyte (TIL) expressing an exogenous tethered IL-12 (TeIL-12), and an exogenous tethered IL-15 (TeIL-15), wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62, and wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.

**[0029]** In some embodiments, the TeIL-12 comprises a membrane anchor, and a human IL-12 p40 subunit fused to a human IL-12 p35 subunit. In some embodiments, the human IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60 and the human IL-12 p40 subunit has the amino acid sequence of SEQ ID NO:61. In some embodiments, the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62. In some embodiments, the gene-edited TIL further expresses a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNF $\alpha$ , IFN alpha, IFN beta, GM-CSF, GCSF, and a variant thereof. In some embodiments, the cytokine is IL-15 or a variant thereof. In some embodiments, the IL-15 is a human IL-15. In some embodiments, the human IL-15 has the amino acid sequence of SEQ ID NO:64. In some embodiments, the IL-15 is a tethered IL-15 (TeIL-15). In some embodiments, the TeIL-15 comprises a membrane anchor, and a human IL-15. In some embodiments, the TeIL-15 has the amino acid sequence of SEQ ID NO:73.

[0030] In some embodiments, the gene-edited TIL comprises a nucleic acid molecule disclosed herein. In some embodiments, the gene-edited TIL comprises a recombinant lentiviral particle disclosed herein. In some embodiments, the gene-edited TIL comprises a recombinant lentiviral RNA molecule disclosed herein. In some embodiments, the gene-edited TIL comprises a recombinant lentiviral proviral DNA disclosed herein. In some embodiments, the recombinant lentiviral proviral DNA is integrated into the genome of the gene-edited TIL.

[0031] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising culturing the population of TILs in a cell culture medium, wherein the cell culture medium comprises IL-15, IL-21, and L-Arginine.

[0032] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising culturing the population of TILs in a cell culture medium, wherein the cell culture medium comprises IL-15, IL-21, L-Arginine, and NAD<sup>+</sup>.

[0033] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium for about 3-14 days; b) performing a second expansion by culturing the population of TILs in a second cell culture medium for about 7-14 days, wherein the second cell culture medium comprises IL-15, IL-21, and L-Arginine.

[0034] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium for about 3-14 days; b) performing a second expansion by culturing the population of TILs in a second cell culture medium for about 7-14 days, wherein the second cell culture medium comprises IL-15, IL-21, L-Arginine, and NAD<sup>+</sup>.

[0035] Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium; and

c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs.

**[0036]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises IL-15 and IL-21; and c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs.

**[0037]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises IL-15, IL-21, and L-Arginine; and c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs.

**[0038]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium for about 7-14 days, wherein the second cell culture medium comprises IL-15, IL-21, L-Arginine, and NAD<sup>+</sup>; and c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs.

**[0039]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) transducing the population of TILs with the recombinant lentiviral particle disclosed herein; and c) performing a second expansion by culturing the population of TILs in a second cell culture medium to produce the population of gene-edited TILs.

**[0040]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a

first expansion by culturing a population of TILs in a first cell culture medium; b) transducing the population of TILs with a recombinant lentiviral particle comprising a recombinant lentiviral RNA molecule comprising a nucleotide encoding a TeIL-12; and c) performing a second expansion by culturing the population of TILs in a second cell culture medium to produce the population of gene-edited TILs.

**[0041]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) transducing the population of TILs with a recombinant lentiviral particle comprising a recombinant lentiviral RNA molecule comprising a nucleotide encoding a TeIL-12 and a nucleotide encoding a TeIL-15; and c) performing a second expansion by culturing the population of TILs in a second cell culture medium to produce the population of gene-edited TILs.

**[0042]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium; and c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs, wherein the population of gene-edited TILs expresses a TeIL-12.

**[0043]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium; and c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs, wherein the population of gene-edited TILs expresses a TeIL-12, and wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.

**[0044]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium; and

c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs, wherein the population of gene-edited TILs expresses a TeIL-12 and a TeIL-15, wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62, and wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.

**[0045]** In some embodiments, the method further comprises activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with a cytokine selected from the group consisting of IL-2, IL-15, IL-21, IL-7, and a combination thereof. In some embodiments, the activating step comprises contacting the population of TILs with 20ng/mL IL-15. In some embodiments, the activating step comprises contacting the population of TILs with 10 ng/mL IL-7. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100. In some embodiments, the transducing step is conducted in the presence of RetroNectin or Vectofusin-1. In some embodiments, the transducing step comprises centrifugation. In some embodiments, the transducing step is conducted in the presence of Lentibooster. In some embodiments, the transducing step is conducted after the first expansion step and before the second expansion step. In some embodiments, the activating step is conducted after the first expansion step and before the second expansion step. In some embodiments, the method further comprises resting the population of TILs for 2 day or 3 days after the transducing step.

**[0046]** In some embodiments, the population of TILs is obtained from a tumor digest. In some embodiments, the method further comprises a priming step. In some embodiments, the priming step is performed before the first expansion step. In some embodiments, the priming step is performed in the presence of IFN $\gamma$ . In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours. In



some embodiments, the method further comprises an enrichment step. In some embodiments, the enrichment step is performed after the priming step and before the first expansion step. In some embodiments, the population of TILs is enriched for CD137+ T cells. In some embodiments, the population of TILs is enriched for CD137+ and CD39+ T cells. In some embodiments, the population of TILs is enriched for CD137+ and CD200+ T cells. In some embodiments, the population of TILs is enriched for CD137+ and OX40+ T cells. In some embodiments, the population of TILs is enriched for CD137+, CD200+ and CD39+ T cells. In some embodiments, the population of TILs is enriched for CD137+, CD200+ and OX40+ T cells. In some embodiments, the population of TILs is enriched for CD137+, CD39+ and OX40+ T cells. In some embodiments, the population of TILs is enriched for CD137+, CD200+, CD39+ and OX40+ T cells. In some embodiments, the first expansion is conducted in the presence of feeder cells. In some embodiments, the feeder cells are T cell-depleted PBMCs. In some embodiments, the first expansion is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion. In some embodiments, the first expansion is performed over a period of about 3-11 days. In some embodiments, the first expansion is performed over a period of about 9 days. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD+ booster. In some embodiments, the NAD+ booster is selected from the group consisting of L-Trp, NR, NMN, NAD+, NAM and P7C3 activator. In some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8. In some embodiments, the second expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 10 days.

[0047] In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-2. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower. In some embodiments, the first cell culture medium or the second cell culture medium contains no added IL-2. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 1 ng/mL to about 100 ng/mL. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 10 ng/mL. In some embodiments, the first cell culture medium comprises IL-2 and IL-21. In some embodiments, the first cell culture medium comprises IL-2 at 3000 IU/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and a protein kinase B (AKT) inhibitor. In some embodiments, the AKT inhibitor is selected from the group consisting of ipatasertib, GSK690693, GSK2141795, GSK2110183, AZD5363, GDC-0068, AT7867, CCT128930, MK-2206, BAY 1125976, Perifosine, Oridonin, Herbacetin, Tehranolide, Isoliquiritigenin, Scutellarin, and Honokiol. In some embodiments, the AKT inhibitor is AZD5363. In some embodiments, the AKT inhibitor is at a concentration of about 0.1  $\mu$ M to about 10  $\mu$ M. In some embodiments, the AKT inhibitor is at a concentration of about 1  $\mu$ M. In some embodiments, the first expansion is performed over a period of about 11 days. In some embodiments, the second expansion is performed over a period of about 11 days.

[0048] Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) priming a tumor digest comprising a population of TILs in the presence of IFN $\gamma$ ; b) enriching the population of TILs for CD137<sup>+</sup> T cells; c) performing a first expansion by culturing the population of TILs enriched for CD137<sup>+</sup> T cells in a first cell culture medium; d) transducing the population of TILs enriched for CD137<sup>+</sup> T cells with a recombinant lentiviral particle comprising a nucleic acid sequence encoding TeIL-12 to produce the population of gene-edited TILs; and e) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium.

[0049] In some embodiments, the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter. In some embodiments, the TeIL-12

comprises a membrane anchor, and a human IL-12 p40 subunit fused to a human IL-12 p35 subunit. In some embodiments, the human IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60 and the human IL-12 p40 subunit has the amino acid sequence of SEQ ID NO:61. In some embodiments, the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62. In some embodiments, the nucleotide sequence encoding the TeIL-12 is set forth in SEQ ID NO:162. In some embodiments, the method further comprises activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100. In some embodiments, the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL. In some embodiments, the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40. In some embodiments, the transducing step is conducted in the presence of RetroNectin or Vectofusin-1. In some embodiments, the transducing step comprises centrifugation. In some embodiments, the transducing step is conducted in the presence of Lentibooster. In some embodiments, the method further comprises resting the population of TILs for 2 day or 3 days after the transducing step. In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours.

**[0050]** In some embodiments, the population of TILs is obtained from a tumor digest. In some embodiments, the method further comprises a priming step. In some embodiments, the priming step is performed before the first expansion step. In some embodiments, the priming step is performed in the presence of IFN $\gamma$ . In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL

during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours. In some embodiments, the first expansion is conducted in the presence of feeder cells. In some embodiments, the feeder cells are T cell-depleted PBMCs. In some embodiments, the first expansion is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion. In some embodiments, the first expansion is performed over a period of about 3-11 days. In some embodiments, the first expansion is performed over a period of about 9 days. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8. In some embodiments, the second expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 10 days.

**[0051]** Some embodiments of the present disclosure provide a method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising: a) priming a tumor digest comprising a population of TILs in the presence of IFN $\gamma$ ; b) enriching the population of TILs for CD137<sup>+</sup> T cells; c) performing a first expansion by culturing the population of TILs enriched for CD137<sup>+</sup> T cells in a first cell culture medium; d) transducing the population of TILs enriched from CD137<sup>+</sup> T cells with a recombinant lentiviral particle comprising a nucleic acid sequence encoding TeIL-12 and TeIL-15 to produce the population

of gene-edited TILs; and e) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium.

**[0052]** In some embodiments, the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter. In some embodiments, the TeIL-15 is under the control of a constitutive promoter. In some embodiments, the constitutive promoter is an EF1 $\alpha$  promoter. In some embodiments, the NFAT promoter and the constitutive promoter are introduced in the same nucleic acid of the coding sequence. In some embodiments, the TeIL-12 comprises a membrane anchor, and a human IL-12 p40 subunit fused to a human IL-12 p35 subunit. In some embodiments, the human IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60 and the human IL-12 p40 subunit has the amino acid sequence of SEQ ID NO:61. In some embodiments, the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62. In some embodiments, the nucleotide sequence encoding the TeIL-12 is set forth in SEQ ID NO:162. In some embodiments, the TeIL-15 comprises an amino acid sequence set forth in SEQ ID NO:73. In some embodiments, the method further comprises activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100. In some embodiments, the method further comprises resting the population of TILs for 2 day or 3 days after the transducing step. In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours.

**[0053]** In some embodiments, the population of TILs is obtained from a tumor digest. In some embodiments, the method further comprises a priming step. In some embodiments, the priming step is performed before the first expansion step. In some embodiments, the priming step is performed in the presence of IFN $\gamma$ . In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step

is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours. In some embodiments, the first expansion is conducted in the presence of feeder cells. In some embodiments, the feeder cells are T cell-depleted PBMCs. In some embodiments, the first expansion is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion. In some embodiments, the first expansion is performed over a period of about 3-11 days. In some embodiments, the first expansion is performed over a period of about 9 days. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8. In some embodiments, the second expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 10 days.

**[0054]** Some embodiments of the present disclosure provide a therapeutic population of TILs produced by a method of making a population of gene-edited TILs disclosed herein. In some embodiments, about 15-60% of the population of TILs expresses the TeIL-12. In some embodiments, more than 30% of the population of TILs expresses the TeIL-12. In some

embodiments, more than 50% of the population of TILs expresses the TeIL-12. In some embodiments, about 15-60% of the population of TILs expresses the TeIL-15. In some embodiments, more than 30% of the population of TILs expresses the TeIL-15. In some embodiments, more than 50% of the population of TILs expresses the TeIL-15. In some embodiments, about 15-60% of the population of TILs expresses the TeIL-2. In some embodiments, more than 30% of the population of TILs expresses the TeIL-2. In some embodiments, more than 50% of the population of TILs expresses the TeIL-2. In some embodiments, more than 30% of the population of TILs has reduced expression of PD-1. In some embodiments, more than 40% of the population of TILs has reduced expression of PD-1. In some embodiments, more than 60% of the population of TILs has reduced expression of PD-1.

**[0055]** Some embodiments of the present disclosure provide a pharmaceutical composition comprising a therapeutic population of TILs disclosed herein.

**[0056]** Some embodiments of the present disclosure provide a method of treating a cancer in a patient in need thereof comprising: a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs; b) performing a first expansion by culturing the population of TILs in a first cell culture medium; c) performing a second expansion by culturing the population of TILs in a second cell culture medium; d) at any time, transducing the population of TILs with a recombinant lentiviral particle disclosed herein to produce a therapeutic population of gene-edited TILs; and e) administering the therapeutic population of gene-edited TILs to the patient. In some embodiments, the method further comprises activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with a cytokine selected from the group consisting of IL-2, IL-15, IL-21, IL-7, and a combination thereof. In some embodiments, the activating step comprises contacting the population of TILs with 20ng/mL IL-15. In some embodiments, the activating step comprises contacting the population of TILs with 10 ng/mL IL-7. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100. In some embodiments, the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL. In some embodiments, the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40. In some embodiments, the transducing step is conducted in the presence of

RetroNectin or Vectofusin-1. In some embodiments, the transducing step comprises centrifugation. In some embodiments, the transducing step is conducted in the presence of Lentibooster. In some embodiments, the transducing step is conducted after the first expansion step and before the second expansion step. In some embodiments, the activating step is conducted after the first expansion step and before the second expansion step. In some embodiments, the method further comprises resting the population of TILs for 2 day or 3 days after the transducing step.

[0057] In some embodiments, the population of TILs is obtained from a tumor digest. In some embodiments, the method further comprises a priming step. In some embodiments, the priming step is performed before the first expansion step. In some embodiments, the priming step is performed in the presence of IFN $\gamma$ . In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours. In some embodiments, the method further comprises an enrichment step. In some embodiments, the enrichment step is performed after the priming step and before the first expansion step. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup> and CD39<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup> and CD200<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup> and OX40<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup>, CD200<sup>+</sup> and CD39<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup>, CD200<sup>+</sup> and OX40<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup>, CD39<sup>+</sup> and OX40<sup>+</sup> T cells. In some embodiments, the population of TILs is enriched for CD137<sup>+</sup>, CD200<sup>+</sup>, CD39<sup>+</sup> and OX40<sup>+</sup> T cells. In some embodiments, the first expansion is conducted in the presence of feeder cells. In some embodiments, the feeder cells are T cell-depleted PBMCs. In some embodiments, the first expansion is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a



concentration of about 1 ng/mL to about 100 ng/mL during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion. In some embodiments, the first expansion is performed over a period of about 3-11 days. In some embodiments, the first expansion is performed over a period of about 9 days. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8. In some embodiments, the second expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 10 days.

**[0058]** In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-2. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower. In some embodiments, the first cell culture medium or the second cell culture medium contains no added IL-2. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 1 ng/mL to about 100 ng/mL. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 10 ng/mL. In some embodiments, the first cell culture medium comprises IL-2 and IL-21. In some embodiments, the first cell culture medium comprises IL-2 at 3000 IU/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and a protein kinase B (AKT) inhibitor. In some embodiments, the AKT inhibitor is selected from the group consisting of ipatasertib, GSK690693, GSK2141795,

GSK2110183, AZD5363, GDC-0068, AT7867, CCT128930, MK-2206, BAY 1125976, Perifosine, Oridonin, Herbacetin, Tehranolide, Isoliquiritigenin, Scutellarin, and Honokiol. In some embodiments, the AKT inhibitor is AZD5363. In some embodiments, the AKT inhibitor is at a concentration of about 0.1  $\mu\text{M}$  to about 10  $\mu\text{M}$ . In some embodiments, the AKT inhibitor is at a concentration of about 1  $\mu\text{M}$ . In some embodiments, the first expansion is performed over a period of about 11 days. In some embodiments, the second expansion is performed over a period of about 11 days.

**[0059]** In some embodiments, the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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 method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60  $\text{mg}/\text{m}^2/\text{day}$  for two days followed by administration of fludarabine at a dose of 25  $\text{mg}/\text{m}^2/\text{day}$  for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60  $\text{mg}/\text{m}^2/\text{day}$  and fludarabine at a dose of 25  $\text{mg}/\text{m}^2/\text{day}$  for two days followed by administration of fludarabine at a dose of 25  $\text{mg}/\text{m}^2/\text{day}$  for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60  $\text{mg}/\text{m}^2/\text{day}$  and fludarabine at a dose of 25  $\text{mg}/\text{m}^2/\text{day}$  for two days followed by administration of fludarabine at a dose of 25  $\text{mg}/\text{m}^2/\text{day}$  for one day. In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient. In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance. In some embodiments, the method comprises no step of treating the patient with an IL-2

regimen. In some embodiments, the therapeutic population of TILs comprises from about  $2.3 \times 10^{10}$  to about  $13.7 \times 10^{10}$  TILs.

**[0060]** Some embodiments of the present disclosure provide a packaging cell line comprising a recombinant expression vector disclosed herein, and one or more recombinant DNA molecules, each of which one or more recombinant DNA molecules encodes any one protein, any two proteins, any three proteins or all four proteins selected from the group consisting of encoding an Env protein, a Gag protein, a Pol protein, and a Rev protein, with the proviso that any protein selected from the group consisting of the Env protein, the Gag protein, the Pol protein and the Rev protein is encoded by at least one recombinant DNA molecule of the one or more recombinant DNA molecules, wherein each of the one or more recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid. In some embodiments, the one or more recombinant DNA molecules consist of a first recombinant DNA molecule. In some embodiments, the first recombinant DNA molecule is either integrated into the genome of the packaging cell line or comprised by a helper plasmid. In some embodiments, the one or more recombinant DNA molecules consist of a first recombinant DNA molecule and a second recombinant DNA molecule. In some embodiments, the first recombinant DNA molecule encodes a single protein selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein. In some embodiments, the first recombinant DNA molecule encodes the Env protein. In some embodiments, the first recombinant DNA molecule encodes the Gag protein. In some embodiments, the first recombinant DNA molecule encodes the Pol protein. In some embodiments, the first recombinant DNA molecule encodes the Rev protein. In some embodiments, the first recombinant DNA molecule encodes two proteins selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein. In some embodiments, the first recombinant DNA molecule encodes the Env protein and the Gag protein. In some embodiments, the first recombinant DNA molecule encodes the Env protein and the Pol protein. In some embodiments, the first recombinant DNA molecule encodes the Env protein and the Rev protein. In some embodiments, the first recombinant DNA molecule encodes the Gag protein and the Pol protein. In some embodiments, the first recombinant DNA molecule encodes the Gag protein and the Rev protein. In some embodiments, the first recombinant DNA molecule encodes the Pol protein and the Rev protein. In some embodiments, each of the first and second recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid.

In some embodiments, the one or more recombinant DNA molecules consist of a first recombinant DNA molecule, a second recombinant DNA molecule, and a third recombinant DNA molecule. In some embodiments, the first recombinant DNA molecule encodes a first protein and the second recombinant DNA molecule encodes a second protein, wherein the first and second proteins are independently selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein, with the proviso that the first and second proteins are not the same. In some embodiments, the first protein is the Env protein and the second protein is the Gag protein. In some embodiments, the first protein is the Env protein and the second protein is the Pol protein. In some embodiments, the first protein is the Env protein and the second protein is the Rev protein. In some embodiments, the first protein is the Gag protein and the second protein is the Pol protein. In some embodiments, the first protein is the Gag protein and the second protein is the Rev protein. In some embodiments, the first protein is the Pol protein and the second protein is the Rev protein. In some embodiments, the first recombinant DNA molecule encodes a first protein and the second recombinant DNA molecule encodes two proteins, wherein the first protein and each of the two proteins are independently selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein, with the proviso that any protein is not the same as any other protein in the group consisting of the first protein and the two proteins. In some embodiments, the first protein is the Env protein, and the two proteins are the Gag protein and the Pol protein. In some embodiments, the first protein is the Env protein, and the two proteins are the Gag protein and the Rev protein. In some embodiments, the first protein is the Env protein, and the two proteins are the Pol protein and the Rev protein. In some embodiments, the first protein is the Gag protein, and the two proteins are the Env protein and the Pol protein. In some embodiments, the first protein is the Gag protein, and the two proteins are the Env protein and the Rev protein. In some embodiments, the first protein is the Gag protein, and the two proteins are the Pol protein and the Rev protein. In some embodiments, the first protein is the Pol protein, and the two proteins are the Env protein and the Gag protein. In some embodiments, the first protein is the Pol protein, and the two proteins are the Env protein and the Rev protein. In some embodiments, the first protein is the Pol protein, and the two proteins are the Gag protein and the Rev protein. In some embodiments, the first protein is the Rev protein, and the two proteins are the Env protein and the Gag protein. In some embodiments, the first protein is the Rev protein, and the two proteins are the Env protein and the Pol protein. In some embodiments, the first protein is the Rev protein, and the two proteins are the Gag protein and the Pol protein. In

some embodiments, each of the first, second, and third recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid. In some embodiments, the one or more recombinant DNA molecules consist of a first recombinant DNA molecule, a second recombinant DNA molecule, a third recombinant DNA molecule, and a fourth recombinant DNA molecule. In some embodiments, each of the first, second, third, and fourth recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid. In some embodiments, the Env protein comprises a Ba-EVTR protein. In some embodiments, the nucleic acid molecule encoding TeIL-12 is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA molecule encoding one or more the Env protein, the Gag protein, the Pol protein, and the Rev protein is under the control of an inducible promoter. In some embodiments, the packaging cell line is a 293T cell line.

**[0061]** Some embodiments of the present disclosure provide a method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising: a) priming a population of TILs obtained from a tumor digest in the presence of IFN $\gamma$ ; b) enriching the population of TILs for CD137<sup>+</sup> TILs; c) performing a first expansion by culturing the population of TILs enriched for CD137<sup>+</sup> T cells in a first cell culture medium; and d) performing a second expansion by culturing the population of TILs enriched for CD137<sup>+</sup> TILs in a second cell culture medium to produce a population of TILs enriched for tumor-reactive TILs. In some embodiments, the population of TILs enriched for tumor-reactive TILs comprises an increased percentage of tumor-reactive TILs in comparison to TILs expanded using a reference expansion procedure.

**[0062]** In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 30 hours. In some embodiments, the first expansion is conducted in the presence of feeder cells. In some embodiments, the feeder cells are T cell-depleted PBMCs. In some embodiments, the first expansion is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the first expansion. In some embodiments, the IL-15 and/or IL-

21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion. In some embodiments, the first expansion is performed over a period of about 3-11 days. In some embodiments, the first expansion is performed over a period of about 9 days. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8. In some embodiments, the second expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 10 days. In some embodiments, the enriching the population of TILs for CD137<sup>+</sup> TILs comprises contacting the population of TILs with anti-CD137 antibody immobilized on a bead. In some embodiments, the method further comprises contacting the population of TILs with an anti-CD39 antibody. In some embodiments, the method further comprises contacting the population of TILs with an anti-CD200 antibody. In some embodiments, the method further comprises contacting the population of TILs with an anti-OX40 antibody.

**[0063]** Some embodiments of the present disclosure provide a method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising: a) priming a population of TILs obtained from a tumor digest in the presence of IFN $\gamma$ ; b) enriching the population of TILs for CD103<sup>+</sup> TILs; c) performing a first expansion by culturing the population of TILs enriched for CD103<sup>+</sup> T cells in a first cell culture medium; and d) performing a second expansion by culturing the population of TILs enriched for CD103<sup>+</sup> T cells in a second cell culture medium to produce a population of TILs enriched for tumor-reactive TILs. In some embodiments, the population of TILs enriched for tumor-reactive TILs comprises an

increased percentage of tumor-reactive TILs in comparison to TILs expanded using a reference expansion procedure.

[0064] In some embodiments, IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step. In some embodiments, the priming step is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step. In some embodiments, the priming step lasts for 24-48 hours. In some embodiments, the priming step lasts for about 24 hours. In some embodiments, selecting CD103<sup>+</sup> TILs comprises sorting the second population of TILs using flow cytometry. In some embodiments, step (b) comprises selecting CD103<sup>+</sup>CD31<sup>-</sup> TILs. In some embodiments, the first expansion is conducted in the presence of feeder cells. In some embodiments, the feeder cells are T cell-depleted PBMCs. In some embodiments, the first expansion is performed in the presence of IL-2, IL-15, and/or IL-21. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the first expansion. In some embodiments, the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion. In some embodiments, the first expansion is performed over a period of about 3-11 days. In some embodiments, the first expansion is performed over a period of about 9 days. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8. In some embodiments, the second expansion is performed over a

period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 10 days. Some embodiments of the present disclosure provide a method of treating a cancer in a patient in need thereof comprising:

- a. resecting a tumor sample from the patient;
- b. digesting the tumor sample to obtain a tumor digest;
- c. producing a population of TILs enriched for tumor-reactive TILs from the tumor digest using the methods disclosed herein; and
- d. administering the therapeutic population of gene-edited TILs to the patient.

**[0065]** In some embodiments, the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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 method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day. In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient. In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof,



administered as a 15-minute bolus intravenous infusion every eight hours until tolerance. In some embodiments, the method comprises no step of treating the patient with an IL-2 regimen. In some embodiments, the therapeutic population of TILs comprises from about  $2.3 \times 10^{10}$  to about  $13.7 \times 10^{10}$  TILs.

**[0066]** Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising:

- a) performing a first expansion by culturing a population of TILs in a first cell culture medium; and
- b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, and L-arginine.

**[0067]** Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising:

- a) performing a first expansion by culturing a population of TILs in a first cell culture medium; and
- b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, L-arginine, and NAD<sup>+</sup>.

**[0068]** In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the NAD<sup>+</sup> booster is NAD<sup>+</sup>. In some embodiments, the NAD<sup>+</sup> is present at a concentration of about 50-75  $\mu$ M. In some embodiments, the first expansion is performed over a period of about 3-14 days. In some embodiments, the first expansion is performed over a period of about 11 days. In some embodiments, the second expansion is performed over a period of about 7-14 days. In some embodiments, the second expansion is performed over a period of about 11 days.

**[0069]** Some embodiments of the present disclosure provide a therapeutic population of TILs produced by the methods disclosed herein.

[0070] Some embodiments of the present disclosure provide a pharmaceutical composition comprising the therapeutic population of TILs disclosed herein.

[0071] Some embodiments of the present disclosure provide a method of treating a cancer in a patient in need thereof comprising:

- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
- b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
- c) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, and L-arginine; and
- d) administering the therapeutic population of gene-edited TILs to the patient.

[0072] Some embodiments of the present disclosure provide a method of treating a cancer in a patient in need thereof comprising:

- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
- b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
- c) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, L-arginine, and NAD<sup>+</sup>; and
- d) administering the therapeutic population of gene-edited TILs to the patient.

[0073] In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the NAD<sup>+</sup> booster is NAD<sup>+</sup>. In some embodiments, the NAD<sup>+</sup> is present at a concentration of about 50-75  $\mu$ M. In some embodiments, the first expansion is performed over a period of about 3-14 days. In some

embodiments, the first expansion is performed over a period of about 11 days. In some embodiments, the second expansion is performed over a period of about 7-14 days. In some embodiments, the second expansion is performed over a period of about 11 days.

**[0074]** In some embodiments, the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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 method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day. In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient. In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance. In some embodiments, the method comprises no step of treating the patient with an IL-2 regimen.

**[0075]** Some embodiments of the present disclosure provide a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

- (a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;
- (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs, and wherein the transition from step (a) to step (b) occurs without opening the system;
- (c) performing a second expansion by culturing the second population of TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;
- (d) harvesting the therapeutic population of TILs obtained from step (c), wherein the transition from step (c) to step (d) occurs without opening the system, and wherein the therapeutic population of TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
- (e) transferring the harvested TIL population from step (d) to an infusion bag, wherein the transfer from step (d) to (e) occurs without opening the system, and
- (f) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

**[0076]** Some embodiments of the present disclosure provide a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

- (a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;
- (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable

surface area, wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs, and wherein the transition from step (a) to step (b) occurs without opening the system;

(c) performing a second expansion by culturing the second population of TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) harvesting the therapeutic population of TILs obtained from step (c), wherein the transition from step (c) to step (d) occurs without opening the system, and wherein the therapeutic population of TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

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

(f) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

**[0077]** Some embodiments of the present disclosure provide a method for making a therapeutic population of gene-edited TILs comprising:

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

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

- (c) transducing the second population of TILs with the recombinant lentiviral particle disclosed herein to produce a population of gene-edited TILs, wherein the transition from step (b) to step (c) occurs without opening the system;
- (d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;
- (e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system, and wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
- (f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, wherein the transfer from step (e) to (f) occurs without opening the system, and
- (g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

**[0078]** Some embodiments of the present disclosure provide a method for making a therapeutic population of gene-edited TILs comprising:

- (a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;
- (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs, and wherein the transition from step (a) to step (b) occurs without opening the system;

- (c) transducing the second population of TILs with the recombinant lentiviral particle disclosed herein to produce a population of gene-edited TILs, wherein the transition from step (b) to step (c) occurs without opening the system;
- (d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;
- (e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system, and wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
- (f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, wherein the transfer from step (e) to (f) occurs without opening the system, and
- (g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

**[0079]** Some embodiments of the present disclosure provide a method for making a therapeutic population of gene-edited TILs comprising:

- (a) processing a tumor resected from a patient to obtain a first population of TILs;
- (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, and wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs;
- (c) transducing the second population of TILs with the recombinant lentiviral particle disclosed herein to produce a population of gene-edited TILs;

(d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, and wherein the second expansion is performed in a closed container providing a second gas-permeable surface area;

(e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

(f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, and

(g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

[0080] Some embodiments of the present disclosure provide a method for making a therapeutic population of gene-edited TILs comprising:

(a) processing a tumor resected from a patient to obtain a first population of TILs;

(b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, and wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs;

(c) transducing the second population of TILs with the recombinant lentiviral particle disclosed herein to produce a population of gene-edited TILs;

(d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, and wherein the second expansion is performed in a closed container providing a second gas-permeable surface area;



- (e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
- (f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, and
- (g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

[0081] In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the L-arginine is present at a concentration of about 1 mM to about 10 mM. In some embodiments, the L-arginine is present at a concentration of about 5 mM. In some embodiments, the second cell culture medium comprises an NAD<sup>+</sup> booster. In some embodiments, the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator. In some embodiments, the NAD<sup>+</sup> booster is NAD<sup>+</sup>. In some embodiments, the NAD<sup>+</sup> is present at a concentration of about 50-75  $\mu$ M. In some embodiments, the first expansion is performed over a period of about 3-14 days. In some embodiments, the first expansion is performed over a period of about 11 days. In some embodiments, the second expansion is performed over a period of about 7-14 days. In some embodiments, the second expansion is performed over a period of about 11 days.

[0082] In some embodiments, the method further comprises activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100. In some embodiments, the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL. In some embodiments, the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40. In some embodiments, the transducing step is conducted in the presence of RetroNectin or Vectofusin-1. In some embodiments, the transducing step comprises centrifugation. In some embodiments, the transducing step is conducted in the presence of Lentibooster. In some embodiments, the method further comprises resting the population of TILs for 2 day or 3 days after the transducing step.

[0083] Some embodiments of the present disclosure provide a therapeutic population of gene-edited TILs produced by the methods disclosed herein.

[0084] Some embodiments of the present disclosure provide a pharmaceutical composition comprising the therapeutic population of gene-edited TILs disclosed herein.

[0085] Some embodiments of the present disclosure provide a use of the therapeutic population of TILs disclosed herein, or the therapeutic population of gene-edited TILs disclosed herein, for treating cancer in a patient in need thereof, comprising administering the therapeutic population of TILs or the therapeutic population of gene-edited TILs to the patient.

[0086] In some embodiments, the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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 method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day. In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient. In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof,

administered as a 15-minute bolus intravenous infusion every eight hours until tolerance. In some embodiments, the method comprises no step of treating the patient with an IL-2 regimen.

#### ***BRIEF DESCRIPTION OF THE DRAWINGS***

[0087] **Figure 1A-1C:** Summary of study to assess expression and signaling of membrane bound IL-15/IL-21 transduced pre-REP TILs.

[0088] **Figure 2A-2B:** Summary of study to assess expression of mIL-15/IL21 and CD8 and CD4 T cell subset in mIL-15/IL-21 transduced REP TILs.

[0089] **Figure 3A-3C:** Summary of study to assess phenotype of mIL-15/IL-21 transduced CD8+ REP TILs.

[0090] **Figure 4A-4C:** Summary of study to assess phenotype of mIL-15/IL-21 transduced CD4+ REP TILs.

[0091] **Figure 5** depicts an exemplary nucleic acid that allows for expression of a member anchored IL-12 (TeIL-12) and PD-1 shRNA in embodiments of the subject TILs provided herein.

[0092] **Figure 6** depicts an exemplary workflow for the preparation of TILs expressing TeIL-12 and/or NFAT-TeIL-12 for administration to a subject.

[0093] **Figure 7A-7B:** Summary of study to assess the expression of TeIL-12 and/or NFAT-TeIL-12 in REP TILs. (A) After REP harvest, surface expression of TeIL-12 on TeIL12 TIL was examined by flow assay with IL-12P70 flow Ab (B). NFAT-TeIL-12 transduced REP-TIL was stimulated with TransACT with indicated dilution or PMA. 48 hours after stimulation, surface expression TeIL-12 expression was examined.

[0094] **Figure 8:** Summary of a study to assess IL-12 activity in TeIL-12 expressing TILs.

[0095] **Figure 9A-9B:** Summary of study to assess (A) expansion and (B) viability of post-REP TILs transduced to express TeIL-12 or NFAT-TeIL-12.

[0096] **Figure 10A-10B:** Summary of study to assess (A) the frequency of TeIL-12 and/or NFAT-TeIL-12 in a population of REP TILs from various tissues (including two lung, one head & neck, one breast and one ovarian tumor samples) and (B) viral genome copy number (VCN) per cell.

[0097] **Figure 11A-11D:** Summary of study to assess (A) and (B) cytotoxicity in a THP-1 based allogenic cytotoxicity assay, (C) and (D) IFN- $\gamma$  production of TeIL-12 REP-TILs and NFAT-driven inducible TeIL-12 REP-TILs.

[0098] **Figure 12A-12B:** Summary of study to assess cytotoxicity of TeIL-12 expressing TILs was also assessed by xCelligence RTCA assay using two target cell populations (A) and (B).

[0099] **Figure 13A-13C:** Summary of a study to assess TIL killing efficacy. (A) Schematic of experimental design. (B) KILR® THP-1 cytotoxicity assay and IFN- $\gamma$  quantification, and (C) Xcelligence RTCA killing assay were performed.

[00100] **Figure 14** depicts a summary of study to assess distribution of CD8<sup>+</sup>, CD4<sup>+</sup>, and CD4<sup>+</sup>/FoxP3<sup>+</sup> T cells within a population of REP TILs transduced to express TeIL-12 or NFAT-TeIL-12.

[00101] **Figure 15A-15B:** Summary of study to assess T cell differentiation of (A) CD8<sup>+</sup> and (B) CD4<sup>+</sup> T cells, as measured by various cellular markers, within a population of REP TILs transduced to express TeIL-12 or NFAT-TeIL-12.

[00102] **Figure 16A-16B:** Summary of study to assess T cell exhaustion of (A) CD8<sup>+</sup> and (B) CD4<sup>+</sup> T cells, as measured by various cellular markers, within a population of REP TILs transduced to express TeIL-12 or NFAT-TeIL-12.

[00103] **Figure 17A-17B:** Summary of study to assess T cell activation of (A) CD8<sup>+</sup> and (B) CD4<sup>+</sup> T cells, as measured by various cellular markers, within a population of REP TILs transduced to express TeIL-12 or NFAT-TeIL-12.

[00104] **Figure 18A-18B:** Summary of study to assess T cell function of (A) CD8<sup>+</sup> and (B) CD4<sup>+</sup> T cells, as measured by various cellular markers, within a population of REP TILs transduced to express TeIL-12 or NFAT-TeIL-12.

[00105] **Figure 19A-19B:** Shows (A) cell expansion and (B) surface expression of TeIL-15 after the procedure: after gene transduction of TeIL-15 lentivirus, Pre-REP TIL was processed for REP expansion with feeder cell, 3000 IU/ml IL-2 and  $\alpha$ CD3 Ab OKT3 or HIT3a. OKT3 (30 ng/ml) or HIT3a (30 ng/ml) was added into REP culture medium in different days (Day 0, Day 2 and Day 4) after setting REP process. After 11 days REP expansion, Post-REP-TIL was harvested and analyzed.

[00106] **Figure 20A-20B:** Shows (A) cell expansion and (B) surface expression of TeIL-15/TeIL-21 after the procedure: after gene transduction of TeIL-15/TeIL-21 lentivirus, Pre-REP TIL was processed for REP expansion with feeder cell, 3000IU/ml IL-2 and aCD3 Ab OKT3 or HIT3a. OKT3 (30ng/ml) or HIT3a(30ng/ml) was added into REP culture medium in different days ( Day 0, Day 2 and Day 4) after setting REP process. After 11 days REP expansion, Post-REP-TIL was harvested and analyzed.

[00107] **Figure 21A-21B:** Shows (A) cell expansion and (B) surface expression of TeIL-15 after the procedure: after gene transduction of TeIL-15 lentivirus, Pre-REP TIL was processed for 11 days REP expansion with feeder cell, 3000IU/ml IL-2 and OKT3 with indicated concentration. After 11 days REP expansion, Post-REP-TIL was harvested and analyzed.

[00108] **Figure 22A-22B:** Shows (A) cell expansion and (B)surface expression of TeIL-15/TeIL-21 after the procedure: after gene transduction of TeIL-15/TeIL-21 lentivirus, Pre-REP TIL was processed for REP expansion with feeder cell, 3000IU/ml IL-2 and OKT3 with indicated concentration. After 11 days REP expansion, Post-REP-TIL was harvested and analyzed.

[00109] **Figure 23:** Shows surface expression of TeIL-12 after transduction with or without TransAct.

[00110] **Figure 24:** Shows surface expression of TeIL-12 after transduction using Retronectin or Vectofusin coated plates, and with or without centrifugation.

[00111] **Figure 25:** Shows surface expression of TeIL-12 after transduction using TransACT or PMA-Inomycin stimulation, and with or without Lentiboost.

[00112] **Figure 26:** Shows surface expression of TeIL-12 after transduction using the BaEVTR, RD114 or VSV-G Env plasmid.

[00113] **Figure 27A-27D:** Shows results from (A) KILR® THP-1 cell killing assay, (B) IFN production, (C) xCelligence RTCA assay, and (D) xCelligence RTCA assay after serial TransACT stimulation, of NFAT-TeIL-12 expressing TILs.

[00114] **Figure 28:** Shows an exemplary expression vector encoding NFAT driven TeIL-12 and EF1 $\alpha$  driven bicistronic tCD19 and Component X.

[00115] **Figure 29A-29B:** Shows (A) fold expansion and (B) viability of TILs transduced with NFAT-TeIL-12 and Component X.

- [00116] **Figure 30A-30B:** Shows (A) transduction efficiency and (B) viral copy number per cell (VCN) of TILs transduced with NFAT-TeIL-12 and Component X.
- [00117] **Figure 31:** Shows expression of tCD19, TeIL-2, TeIL-15, IL-2, IL-15, GFP shRNA and PD-1 shRNA after different levels of TCR stimulation.
- [00118] **Figure 32:** Shows results from xCELLgene RTCA cell killing assay of TILs expressing tCD19, TeIL-2, TeIL-15, IL-2, or IL-15.
- [00119] **Figure 33:** Shows p-STAT activation induced by TILs expressing TeIL-2, TeIL-15, IL-2, or IL-15.
- [00120] **Figure 34:** Shows skewed in cis proliferation of TILs expressing TeIL-2, TeIL-15, IL-2, or IL-15.
- [00121] **Figure 35A-35B:** Shows EF-1 $\alpha$  driven IL-2/IL-15 improve T cell proliferation in the absence of IL-2.
- [00122] **Figure 36A-36B:** Shows PD-1 shRNA effectively reduces PD-1 positivity in TILs measured by flow cytometry (A) or gMFI (B).
- [00123] **Figure 37A-37D:** Shows TeIL-15 facilitates TIL survival in vitro in the absence of IL-2.
- [00124] **Figure 38A-38B:** Shows PDX data of in vivo efficacy of NFAT-IL-12 gene engineered TIL using Gen 2 or Invigo-T + I-Arg processes.
- [00125] **Figure 39A-39B:** Shows dynamic change of body weight (A) and body weight % related baseline (B) of NFAT-IL-12 gene engineered TIL using Gen 2 or Invigo-T + L-Arg processes.
- [00126] **Figure 40:** Shows plasma level of IFN- $\gamma$ , TNF $\alpha$ , CCL4 and IL-12 p70 in mice infused with Gen 2 or NFAT-IL-12 gene engineered TILs.
- [00127] **Figure 41:** Shows tumor reactivity was enhanced when TILs were cultured with ITIL cytokines in the presence of IFN $\gamma$ .
- [00128] **Figure 42A-42B:** Shows 4-1BB expression was found to be maximal 24 hours after plating the tumor digest (A), and increase with the provision of IFN $\gamma$  to the cell culture media (TS-TIL condition) (B).
- [00129] **Figure 43:** Outlines several processes that were analyzed for their capacity to enrich tumor-reactive CD8 TILs.

[00130] **Figure 44:** Shows ~10x improvement in product potency of TILs made by the CD137 sorted process.

[00131] **Figure 45:** Shows enrichment for tumor reactive TILs made by the TS-TIL (4-1BB sort/1x REP) process.

[00132] **Figure 46:** Shows both CD137 (4-1BB) sorting conditions provided the best enrichment for tumor-reactive CD8s (IFN $\gamma$  +CD107a+) TILs.

[00133] **Figure 47:** Shows 60-fold improvement for tumor-reactive CD8s (IFN $\gamma$  +CD107a+) TILs by CD137 (4-1BB) sorting condition.

[00134] **Figure 48:** Outlines the “TS-TIL” (4-1BB selection) process for producing tumor reactive TILs.

[00135] **Figure 49:** Shows a significant increase in percentage of specific tumor reactive TILs in the TS-TIL product (~7%) vs the CTRL product (~1%).

[00136] **Figure 50:** Shows the total number of CD8 tumor-reactive TILs generated from the TS-TIL process is, on average 7-fold, greater than the total number of CD8 tumor-reactive TILs generated from the CTRL process.

[00137] **Figure 51:** Shows the results for total viable cells and fold expansion as a function of REP conditions.

[00138] **Figure 52:** Shows the total yield of CD8+ tumor-reactive TIL (as assessed by autologous digest coculture ICS) as a function of sort and REP conditions.

[00139] **Figure 53:** Shows that the majority of cells was found to be Tem “effector memory” cells, with minimal differences among the TILs made from different processes.

[00140] **Figure 54:** Shows that TeIL-15 activated T cells in cis manner only.

[00141] **Figure 55A-55D:** Shows that L-Arginine enhances expansion and cell recovery post thaw during Invigo-T REP.

[00142] **Figure 56A-56C:** Shows that L-Arginine provides better phenotype and killing capacity.

[00143] **Figure 57A-57C:** Shows that NAD<sup>+</sup> synergies with L-Arginine in boosting metabolism and providing a unique T cell signature.

[00144] **Figure 58** shows the TIL manufacturing process from banked and fresh preREP TILs.

[00145] **Figure 59** shows the transduction efficiency in CD3+ T cells for NFAT-TeIL12-EF1 $\alpha$ -tCD19 (mean=30.3%) and NFAT-TeIL12-EF1 $\alpha$ -TeIL15 (mean= 23.2%).

[00146] **Figure 60A** shows the transduction efficiency in CD4+ and CD8+ T cell subsets demonstrating that the expression of tCD19 in TILs transduced with NFAT-TeIL12-EF1 $\alpha$ -tCD19 is similar between CD4+ and CD8+ cells.

[00147] **Figure 60B** shows the expression of TeIL15 in TILs transduced with NFAT-TeIL12-EF1 $\alpha$ -TeIL15 is higher in CD8+ T cells compared to CD4+ T cells.

[00148] **Figure 61** shows that the lentiviral vector transduced TILs exhibit low/minimal skewing of the percentages of CD4+ or CD8+ cells in the REP TIL product.

[00149] **Figure 62** shows that the REP cell culture media conditions exhibit similar transduction efficiency in CD3+ T cells.

[00150] **Figure 63A-63B** shows that the REP cell culture media conditions exhibit similar transduction efficiency in CD4+ and CD8+ T cell subsets.

[00151] **Figure 64** shows that the expansion rate and viability are enhanced with various REP cell culture media conditions.

[00152] **Figure 65** shows that various REP cell culture media conditions increase the number of TILs (TVC) in untransduced TILs.

[00153] **Figure 66** shows that various REP cell culture media conditions increase the viability in untransduced TILs.

[00154] **Figure 67** shows that the expression level for TeIL-15 in REP TILs is enhanced by GEN2+NAD and ITARG+NAD in CD3+ cells (**A**), CD4+ T cells (**B**), and CD8+ T cells (**C**).

[00155] **Figure 68** shows that untransduced TILs (**A, C**) and NFAT-TeIL-12-EF1 $\alpha$ -TeIL-15 TILs (**B, D**) exhibit increased production of IFN $\gamma$  (**A, B**) and TNF $\alpha$  (**C, D**) when cultured with autologous tumor digest (TIL+Digest) compared to TILs that did not receive any stimulation (Unstimulated).

[00156] **Figure 69** shows that untransduced TILs (**A, C**) and NFAT-TeIL12-EF1 $\alpha$ -TeIL15 TILs (**B, D**) exhibit a reduction of IFN $\gamma$  (**A, B**) and TNF $\alpha$  (**C, D**) when cultured with



autologous tumor digest (TIL+Digest) compared to co-cultures incubated with anti-HLA-I and anti-HLA-II (TIL+Digest+HLA Block).

**[00157]** **Figure 70** shows that NFAT-TeIL12-EF1 $\alpha$ -TeIL15 TILs produce more IFN $\gamma$  when cultured with autologous tumor digest (TIL+Digest) compared to untransduced TILs, which is enhanced when the TILs underwent a GEN2+NAD or ITARG+NAD REP **(A)**. NFAT-TeIL12-EF1 $\alpha$ -TeIL15 TILs expanded under GEN2 conditions exhibited a 2.8-fold increase in IFN $\gamma$ , whereas NFAT-TeIL12-EF1 $\alpha$ -TeIL15 GEN2+NAD TILs and NFAT-TeIL12-EF1 $\alpha$ -TeIL15 ITARG+NAD TILs demonstrated a 6.7-fold increase and a 5.1-fold increase in IFN $\gamma$  respectively **(B)**.

**[00158]** **Figure 71** shows NFAT-TeIL12-EF1 $\alpha$ -TeIL15 TILs produce more TNF $\alpha$  when cultured with autologous tumor digest (TIL+Digest) compared to Untransduced TILs, which is enhanced when the TILs underwent a GEN2+NAD or ITARG+NAD REP **(A)**. NFAT-TeIL12-EF1 $\alpha$ -TeIL15 TILs expanded under GEN2 conditions exhibited a 1.3-fold increase in TNF $\alpha$ , compared to NFAT-TeIL12-EF1 $\alpha$ -TeIL15 GEN2+NAD TILs (1.8-fold), NFAT-TeIL12-EF1 $\alpha$ -TeIL15 ITARG TILs (1.7-fold), and NFAT-TeIL12-EF1 $\alpha$ -TeIL15 ITARG+NAD TILs (1.7-fold) **(B)**.

**[00159]** **Figure 72** shows TILs transduced with NFAT-TeIL12-EF1 $\alpha$ -tCD19 or NFAT-TeIL12-EF1 $\alpha$ -TeIL15 exhibit superior cytotoxicity against an autologous melanoma cell line in comparison to Untransduced TILs. The percentage of Caspase3/7+ cells at the 8:1 ET ratio **(A)** and 4:1 ET ratio **(B)** were significantly higher when tumor cells were cultured with autologous NFAT-TeIL12-EF1 $\alpha$ -tCD19 or NFAT-TeIL12-EF1 $\alpha$ -TeIL15 TILs in comparison to untransduced TILs.

**[00160]** **Figure 73** shows NYESO1 TCR-selected T-cells transduced with either the single NFAT-teIL12 construct or the dual NFAT-teIL12-EF1 $\alpha$ -TeIL15 construct demonstrated superior killing efficacy when compared to lentivirus backbone control and mock process control.

**[00161]** **Figure 74** shows **(A)** total viable cells **(B)** fold expansion and **(C)** viability at the end of 22 day REP process and **(D)** viability 1 hour post-thaw of unmodified TIL expanded under various media formulations. Data displayed as mean  $\pm$  SD. Data by Repeated measures Mixed Model, post-hoc Tukey's multiple comparisons test. \*  $p < 0.01$ ; \*\*  $p < 0.001$ , \*\*\*  $p < 0.0001$ . Abbreviations: ITARG, IL-15, IL-21, L-Arginine; NAD<sup>+</sup>, nicotinamide adenine dinucleotide.

[00162] **Figure 75** shows (A) total viable cells (B) fold expansion and (C) viability at the end of 22 day REP process and (D) viability 1 hour post-thaw of tethered-cytokine TIL (NFAT-TeIL12/TeIL15) expanded under various conditions. Data displayed as mean  $\pm$  SD. Data by Repeated measures Mixed Model, post-hoc Tukey's multiple comparisons test. Ns, not significant; \*  $p < 0.01$ ; \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ . Abbreviations: ITARG, IL-15, IL-21, L-Arginine; NAD<sup>+</sup>, nicotinamide adenine dinucleotide.

[00163] **Figure 76** shows a vector map of pCMV-3Tag-4a-BaEV-TR (SEQ ID NO:165).

[00164] **Figure 77** shows a vector map of pLenti- IRES-NFAT-TeIL12-EF1a-TeIL-15 (SEQ ID NO:166).

[00165] **Figure 78.** (A) Total viable cells (B) fold expansion and (C) viability at the end of 22-day REP process of unmodified TIL expanded under various media formulations from 41 donors across various tumor types. Data displayed as mean  $\pm$  SD. Data by Repeated measures Mixed-effects Model, post-hoc Tukey's multiple comparisons test. \*  $p < 0.01$ ; \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ . Abbreviations: ITARG, IL-15, IL-21, L-Arginine; NAD<sup>+</sup>, nicotinamide adenine dinucleotide.

[00166] **Figure 79.** In vitro cytotoxicity of tumor-directed TIL. Assessment of cytotoxicity of KRAS-TCR TIL products from 5 donors against KRAS (HPAC) tumor cells. Antigen-specific TILs were added to respective HPAC cells in a modified media that simulates conditions often observed in solid tumor microenvironment (TME) (with and without IL-2, (A) and (B), respectively). Data displayed as mean  $\pm$  SD. Data by Repeated measures Mixed-effects Model, post-hoc Tukey's multiple comparisons test. \*  $p < 0.01$ ; \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ . Abbreviations: ITARG, IL-15, IL-21, L-Arginine; NAD<sup>+</sup>, nicotinamide adenine dinucleotide.

[00167] **Figure 80.** In vitro cytotoxicity of tumor-directed TIL with membrane-tethered cytokines. Assessment of cytotoxicity of KRAS.mteth.IL12.IL15 TIL products from 5 donors against KRAS (HPAC) tumor cells in a modified media that simulates conditions often observed in solid tumor microenvironment (TME) (with and without IL-2, (A) and (B), respectively). Data displayed as mean  $\pm$  SD. Data by Repeated measures Mixed-effects Model, post-hoc Tukey's multiple comparisons test. \*  $p < 0.01$ ; \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ . Abbreviations: ITARG, IL-15, IL-21, L-Arginine; NAD<sup>+</sup>, nicotinamide adenine dinucleotide.

The level of mteth.IL15 on the surface of KRAS transduced TIL detected using flow cytometry serves as a control for transduction efficiency (C),

[00168] **Figure 81.** TeIL-12/TeIL-15 expressing TILs show trends of increased persistence after infusion.

[00169] **Figure 82.** Exemplary Gen 2 (process 2A) chart providing an overview of Steps A through F.

[00170] **Figure 83A-83C.** Process flow chart of an embodiment of Gen 2 (process 2A) for TIL manufacturing.

## DETAILED DESCRIPTION

### I. Introduction

[00171] Adoptive cell therapy utilizing TILs is an effective approach for inducing tumor regression in various cancers, including leukemias and melanoma. The use of adjuvants that include immunostimulatory agents has been explored to enhance adoptive cell therapies and to extend such therapies to other solid tumors. Co-administration of immunomodulators such as cytokines (*e.g.*, interleukins), however, can lead to undesirably toxicity due to the high dosages required. Thus, supplying such adjuvants at the right time and site appears crucial to avoid such undesirable effects.

[00172] Provided herein are compositions and methods for the treatment of cancers using modified TILs, wherein the modified TILs include one or more immunomodulatory agents (*e.g.*, cytokines) associated with their cell surface. The immunomodulatory agents associated with the TILs provide a localized immunostimulatory effect that can advantageously enhance TIL survival and/or anti-tumor activity in a patient recipient. As such, the compositions and methods disclosed herein provide effective cancer therapies.

### II. Definitions

[00173] 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.

[00174] 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.

[00175] The term “*in vivo*” refers to an event that takes place in a subject’s body.

[00176] 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.

[00177] 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.

[00178] 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.

[00179] 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<sup>+</sup> cytotoxic T cells (lymphocytes), Th1 and Th17 CD4<sup>+</sup> 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.

**[00180]** By “population of cells” (including TILs) herein is meant a number of cells that share common traits. In general, populations generally range from  $1 \times 10^6$  to  $1 \times 10^{10}$  in number, with different TIL populations comprising different numbers. For example, initial growth of primary TILs in the presence of IL-2 results in a population of bulk TILs of roughly  $1 \times 10^8$  cells. REP expansion is generally done to provide populations of  $1.5 \times 10^9$  to  $1.5 \times 10^{10}$  cells for infusion.

**[00181]** By “cryopreserved TILs” herein is meant that TILs, either primary, bulk, or expanded (REP TILs), are treated and stored in the range of about  $-150^\circ\text{C}$  to  $-60^\circ\text{C}$ . 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.

**[00182]** 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.

**[00183]** 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.

**[00184]** 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.

**[00185]** The term “central memory T cell” refers to a subset of T cells that in the human are CD45RO+ and constitutively express CCR7 (CCR7<sup>hi</sup>) and CD62L (CD62<sup>hi</sup>). 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.

**[00186]** 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<sup>lo</sup>) and are heterogeneous or low for CD62L expression (CD62L<sup>lo</sup>). 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- $\gamma$ , 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.

**[00187]** 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.

**[00188]** 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.

**[00189]** 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.

**[00190]** The term “anti-CD3 antibody” refers to an antibody or variant thereof, *e.g.*, a monoclonal antibody and including human, humanized, chimeric or murine antibodies which

are directed against the CD3 receptor in the T cell antigen receptor of mature T cells. Anti-CD3 antibodies include OKT-3, also known as muromonab. Anti-CD3 antibodies also include the UHCT1 clone, also known as T3 and CD3ε. Other anti-CD3 antibodies include, for example, otelixizumab, teplizumab, and visilizumab.

[00191] 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<br>muromonab heavy<br>chain | QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY INPSRGYTNY 60  |
|                                         | NQKFQDKATL TTDKSSSTAY MQLSSLTSED SAVVYCARYY DDHYCLDYWG QGTTLTVSSA 120 |
|                                         | KTTAPSVVPL APVCGGTGGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TTPAVLQSDL 180 |
|                                         | YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG 240 |
|                                         | PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN 300 |
|                                         | STYRVVSVLT VLGQDWLNGK EYCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE 360  |
|                                         | LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTTPV LDSGGSFFLY SKLTVDKSRW 420 |
|                                         | QQGNVFSCSV MHEALHNHYT QKSLSISPGK 450                                  |
| SEQ ID NO:2<br>muromonab light<br>chain | QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQKSG TSPKRWIYDT SKLASGVPAH 60   |
|                                         | FRSGSGTSY SLTISGMEAE DAATYYCQOW SSNPFTFGSG TKLEINRADT APTVSIFPPS 120  |
|                                         | SEQLTSGGAS VVCFLNMFYP KDINVWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLT 180   |
|                                         | TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC 213                              |

[00192] 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 N<sup>6</sup> 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.

**[00193]** 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-GlcNAc-serine, L-phosphoserine, phosphoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selenyl)propanoic acid, 2-amino-3-(phenylselenyl)propanoic, or selenocysteine. In some embodiments, the IL-2 conjugate has a decreased affinity to IL-2 receptor  $\alpha$  (IL-2R $\alpha$ ) 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 $\alpha$  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 $\alpha$ . 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( $\alpha$ -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), bismaleimido-hexane (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- $[\beta$ -(4-azidosalicylamido)ethyl]disulfide (BASED), formaldehyde, glutaraldehyde, 1,4-butanediol diglycidyl ether, adipic acid dihydrazide, carbonyldiimidazole, o-toluidine, 3,3'-dimethylbenzidine, benzidine,  $\alpha,\alpha'$ -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), succinimidyl- $\alpha$ -methyl- $\alpha$ -(2-pyridyldithio)toluene (sMPT), sulfosuccinimidyl-6-[ $\alpha$ -methyl- $\alpha$ -(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-( $\gamma$ -maleimidobutyryloxy)succinimide ester (GMBs), N-( $\gamma$ -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-carboxylate (sIACX), p-nitrophenyl iodoacetate (NPiA), carbonyl-reactive and thiol-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-methylcoumarin-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.

**[00194]** 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 (Cys<sup>125</sup>>Ser<sup>51</sup>), fused via peptidyl linker (<sup>60</sup>GG<sup>61</sup>) to human interleukin 2 fragment (62-132), fused via peptidyl linker (<sup>133</sup>GSGGGS<sup>138</sup>) to human interleukin 2 receptor  $\alpha$ -chain fragment (139-303), produced in Chinese hamster ovary (CHO) cells, glycosylated; human interleukin 2 (IL-2) (75-133)-peptide [Cys<sup>125</sup>(51)>Ser]-mutant (1-59), fused via a G<sub>2</sub> peptide linker (60-61) to human interleukin 2 (IL-2) (4-74)-peptide (62-132) and via a GSG<sub>3</sub>S peptide linker (133-138) to human interleukin 2 receptor  $\alpha$ -chain (IL2R subunit alpha, IL2R $\alpha$ , 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 $\alpha$  or a protein having at least 98% amino acid sequence identity to IL-1R $\alpha$  and having the receptor antagonist activity of IL-R $\alpha$ , 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<br>recombinant<br>human IL-2<br>(rhIL-2) | MAPTSSSTKK TQLQLEHLL DLQMIINGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL 60<br>EELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN 120<br>RWITFCQSI STLT 134                                                                                                                                                                                                               |
| SEQ ID NO:4<br>Aldesleukin                           | PTSSSTKKTK QLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE 60<br>ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGS ETTFMCEYAD ETATIVEFLNRW 120<br>ITFSQSIIST LT 132                                                                                                                                                                                                               |
| SEQ ID NO:5<br>IL-2 form                             | APTSSSTKKT QLQLEHLLD LQMILNGINN YKNPKLTRML TFKFYMPKKA TELKHLQCLE 60<br>EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGS ETTFMCEYAD ETATIVEFLNR 120<br>WITFCQSIIS TLT 133                                                                                                                                                                                                              |
| SEQ ID NO:6<br>Nemvaleukin alfa                      | SKNFHLRPRD LISNINVIV ELKGSETTFM CEYADETATI VEFLNRWITF SQSIISTLTG 60<br>GSSSTKKTL QLEHLLDLQ MILNGINNYK NPKLTRMLTF KFYMPKKATE LKHLQCLEEE 120<br>LKPLEEVLNL AQSGGGSEL CDDDPPEIPE ATFKAMAYKE GTMLNCECKR GFRRIKSGSL 180<br>YMLCTGNSSH SSWDNQCQCT SSATRNITTK VTPQPEEQKE RKTTEMQSPM QPVDQASLFG 240<br>HCREPPPWEN EATERIYHFV VGQMVVYQCV QGYRALHRGP AESVCKMTHG KTRWTQPQLI 300<br>CTG 303 |
| SEQ ID NO:7<br>IL-2 form                             | MDAMKRGLCC VLLCGAVFV SARPSGRKS SKMQAFRIWD VNOKTFYLRN NQLVAGYLOG 60<br>PNVNLEEKID VVPIEPHALF LGIHGGKMCL SCVKSGETR LQLEAVNITD LSENKQDKR 120<br>FAFIRSDSGP TTSFESAACP GWFLCTAMEA DQPVSLTNMP DEGVMTKFY FQEDSGSGG 180<br>ASSESSASSD GPHPVITESR ASSESSASSD GPHPVITESR EPKSSDKTHT CPPCPAPELL 240                                                                                       |

|                                                         |                                                                                                                                                                                                                                                   |                          |
|---------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|
|                                                         | GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ<br>YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR<br>BEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTP PVLDSGGSFF LYSKLTVDKS<br>RWQQGNVFSC SVMHEALHNH YTKQSLSLSP GK | 300<br>360<br>420<br>452 |
| SEQ ID NO:8<br>mucin domain<br>polypeptide              | SESSASSDGP HPVITP                                                                                                                                                                                                                                 | 16                       |
| SEQ ID NO:9<br>recombinant<br>human IL-4<br>(rhIL-4)    | MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCAA TVLRQFYSHH<br>EKDTRCLGAT AQQFHRKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLRTI<br>MREKYSKCSS                                                                                                | 60<br>120<br>130         |
| SEQ ID NO:10<br>recombinant<br>human IL-7<br>(rhIL-7)   | MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHCDA NKEGMFLFRA<br>ARKLRQFLKM NSTGDFDLHL LKVSEGTIIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL<br>KEQKLNLDLC FLKRLLEIK TCWNKILMGT KEH                                                                      | 60<br>120<br>153         |
| SEQ ID NO:11<br>recombinant<br>human IL-15<br>(rhIL-15) | MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCVKTA MKCFLELQV ISLESGDASI<br>HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNKEF LQSFVHVQM FINTS                                                                                                                    | 60<br>115                |
| SEQ ID NO:12<br>recombinant<br>human IL-21<br>(rhIL-21) | MQDRHMIRMRL QLIDIVDQLK NYVNDIVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG<br>NNERIINVTI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLQKMIHQ<br>HLSSRTHGSE DS                                                                                           | 60<br>120<br>132         |

[00195] 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<sub>H</sub>), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V<sub>L</sub>), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V<sub>H</sub> or the V<sub>L</sub>, 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<sub>H</sub>), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V<sub>L</sub>), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V<sub>H</sub> or the V<sub>L</sub>, 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<sub>H</sub>), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V<sub>L</sub>), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V<sub>H</sub> or the V<sub>L</sub>, 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.

**[00196]** In some embodiments, an IL-2 molecule or a fragment thereof is grafted into HCDR1 of the V<sub>H</sub>, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is grafted into HCDR2 of the V<sub>H</sub>, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is grafted into HCDR3 of the V<sub>H</sub>, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is grafted into LCDR1 of the V<sub>L</sub>, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is grafted into LCDR2 of the V<sub>L</sub>, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is grafted into LCDR3 of the V<sub>L</sub>, wherein the IL-2 molecule is a mutein.

**[00197]** 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 grafted 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 grafted 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.

**[00198]** In some embodiments, an IL-2 molecule is grafted 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 grafted indirectly into a CDR with a peptide linker, with one or more additional amino acids between the CDR sequence and the IL-2 sequence.

**[00199]** 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.

[00200] 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<sub>H</sub> 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<sub>L</sub> 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<sub>H</sub> region comprising the amino acid sequence of SEQ ID NO:28 and a V<sub>L</sub> 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<br>IL-2                  | MYRMQLLS                                 | CI         | ALSLALVTNS | APTSSSTKKT  | QLQLEHLLLD  | LQMILNGINN | YKNPKLTRML | 60  |
|                                       | TFKFYMPKKA                               | TELKHLQC   | LE         | EELKPLEEVL  | NLAQSKNFHL  | RPRDLISNIN | VIVLELKGSE | 120 |
|                                       | TTFMCEYADE                               | TATIVEFLNR | WITFCQSIIS | TLT 153     |             |            |            |     |
| SEQ ID NO:14<br>IL-2 mutein           | APTSSSTKKT                               | QLQLEHLLLD | LQMILNGINN | YKNPKLTAML  | TFKFYMPKKA  | TELKHLQC   | LE         | 60  |
|                                       | EELKPLEEVL                               | NLAQSKNFHL | RPRDLISNIN | VIVLELKGSE  | TTFMCEYADE  | TATIVEFLNR |            | 120 |
|                                       | WITFCQSIIS                               | TLT 133    |            |             |             |            |            |     |
| SEQ ID NO:15<br>IL-2 mutein           | APTSSSTKKT                               | QLQLEHLLLD | LQMILNGINN | YKNPKLTRML  | TAKFYMPKKA  | TELKHLQC   | LE         | 60  |
|                                       | EELKPLEEVL                               | NLAQSKNFHL | RPRDLISNIN | VIVLELKGSE  | TTFMCEYADE  | TATIVEFLNR |            | 120 |
|                                       | WITFCQSIIS                               | TLT 133    |            |             |             |            |            |     |
| SEQ ID NO:16<br>HCDR1_IL-2            | GFSLAPTSSS                               | TKKTQLQLEH | LLDLQMLN   | GINNYKNPKL  | TAMLT       | TFKFYM     | PKKATELKH  | 60  |
|                                       | QCLEEELKPL                               | EEVLNLAQSK | NFHLRPRDLI | SNINVIVLEL  | KGSET       | TFMCE      | YADETATIVE | 120 |
|                                       | FLNRWITFCQ                               | SIISTLTSTS | GMSVG      | 145         |             |            |            |     |
| SEQ ID NO:17<br>HCDR2                 | DIWWDDKKDY                               | NPSLKS     | 16         |             |             |            |            |     |
| SEQ ID NO:18<br>HCDR3                 | SMITNWFYDV                               | 10         |            |             |             |            |            |     |
| SEQ ID NO:19<br>HCDR1_IL-2 kabat      | APTSSSTKKT                               | QLQLEHLLLD | LQMILNGINN | YKNPKLTAML  | TFKFYMPKKA  | TELKHLQC   | LE         | 60  |
|                                       | EELKPLEEVL                               | NLAQSKNFHL | RPRDLISNIN | VIVLELKGSE  | TTFMCEYADE  | TATIVEFLNR |            | 120 |
|                                       | WITFCQSIIS                               | TLTSTSGMSV | G          | 141         |             |            |            |     |
| SEQ ID NO:20<br>HCDR2 kabat           | DIWWDDKKDY                               | NPSLKS     | 16         |             |             |            |            |     |
| SEQ ID NO:21<br>HCDR3 kabat           | SMITNWFYDV                               | 10         |            |             |             |            |            |     |
| SEQ ID NO:22<br>HCDR1_IL-2<br>clothia | GFSLAPTSSS                               | TKKTQLQLEH | LLDLQMLN   | GINNYKNPKL  | TAMLT       | TFKFYM     | PKKATELKH  | 60  |
|                                       | QCLEEELKPL                               | EEVLNLAQSK | NFHLRPRDLI | SNINVIVLEL  | KGSET       | TFMCE      | YADETATIVE | 120 |
|                                       | FLNRWITFCQ                               | SIISTLTSTS | GM         | 142         |             |            |            |     |
| SEQ ID NO:23<br>HCDR2 clothia         | WWDDK                                    |            |            |             |             |            |            | 5   |
| SEQ ID NO:24<br>HCDR3 clothia         | SMITNWFYDV                               | 10         |            |             |             |            |            |     |
| SEQ ID NO:25<br>HCDR1_IL-2 IMGT       | GFSLAPTSSS                               | TKKTQLQLEH | LLDLQMLN   | GINNYKNPKL  | TAMLT       | TFKFYM     | PKKATELKH  | 60  |
|                                       | QCLEEELKPL                               | EEVLNLAQSK | NFHLRPRDLI | SNINVIVLEL  | KGSET       | TFMCE      | YADETATIVE | 120 |
|                                       | FLNRWITFCQ                               | SIISTLTSTS | GMS        | 143         |             |            |            |     |
| SEQ ID NO:26<br>HCDR2 IMGT            | IWWDDKK                                  |            |            |             |             |            |            | 7   |
| SEQ ID NO:27<br>HCDR3 IMGT            | ARSMITNWFY                               | DV         | 12         |             |             |            |            |     |
| SEQ ID NO:28<br>V <sub>H</sub>        | QVTLRESGPA                               | LVKPTQTTL  | TCTFSGFSLA | PTSSSTKKKTQ | LQLEHLLLDL  | QMILNGINNY |            | 60  |
|                                       | KNPKLTAMLT                               | FKFYMPKKAT | ELKHLQCLEE | ELKPLEEVLN  | LAQSKNFHLR  | PRDLISNIN  |            | 120 |
|                                       | IVLELKGSET                               | TFMCEYADET | ATIVEFLNRW | ITFCQSIIST  | LTSTSGMSVG  | WIRQPPGKAL |            | 180 |
|                                       | EWLADIWDD                                | KKDYNPSLKS | RLTISKDTSK | NQVVLKVTNM  | DPADTATYYC  | ARSMITNWFY |            | 240 |
|                                       | DVWGAGTTVT                               | VSS 253    |            |             |             |            |            |     |
| SEQ ID NO:29<br>Heavy chain           | QMILNGINNY                               | KNPKLTAMLT | FKFYMPKKAT | ELKHLQCLEE  | ELKPLEEVLN  | LAQSKNFHLR |            | 60  |
|                                       | PRDLISNIN                                | IVLELKGSET | TFMCEYADET | ATIVEFLNRW  | ITFCQSIIST  | LTSTSGMSVG |            | 120 |
|                                       | WIRQPPGKAL                               | EWLADIWDD  | KKDYNPSLKS | RLTISKDTSK  | NQVVLKVTNM  | DPADTATYYC |            | 180 |
|                                       | ARSMITNWFY                               | DVWGAGTTVT | VSSASTKGPS | VFPLAPSSKS  | TSGGTAAALGC | LVKDYFPEPV |            | 240 |
|                                       | TVSWNSGALT                               | SGVHTFPAVL | QSSGLYSLSS | VVTVPSSSLG  | TQTYICNVNH  | KPSNTKVDKR |            | 300 |
|                                       | VEPKSCDKTE                               | TCPPCPAPEL | LGGPSVFLFP | PKPKDTLMIS  | RTPEVTCVVV  | AVSHEDPEVK |            | 360 |
|                                       | FNWYVDGVEV                               | HNAKTKPREE | QYNSTYRVVS | VLTVLEQDWL  | NGKEYKCKVS  | NKALAAPIEK |            | 420 |

|                |            |            |            |             |            |            |     |
|----------------|------------|------------|------------|-------------|------------|------------|-----|
|                | TISKAKGQPR | EPQVYTLPPS | REEMTKNQVS | LTCLVKGFYP  | SDIAVEWESN | GQPENNYKTT | 480 |
|                | PPVLDSGGSF | FLYSKLTVDK | SRWQQGNVFS | CSVMHEALHN  | HYTQKSLSL  | PGK        | 533 |
|                | KAQLSVGYME | 10         |            |             |            |            |     |
| SEQ ID NO:30   |            |            |            |             |            |            |     |
| LCDR1 kabat    |            |            |            |             |            |            |     |
| SEQ ID NO:31   | DTSKLAS    |            |            |             |            |            | 7   |
| LCDR2 kabat    |            |            |            |             |            |            |     |
| SEQ ID NO:32   | FQSGGYPFT  | 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 | LSASVGDRT  | ITCKAQLSVG | YMHWYQKPG   | KAPKLLIYDT | SKLASGVPSR | 60  |
| V <sub>L</sub> | FSGSGSGTEF | TLTISSLQPD | DFATYYCFQG | SGYPFTFGGG  | TKLEIKRTVA | 106        |     |
| SEQ ID NO:37   | DIQMTQSPST | LSASVGDRT  | ITCKAQLSVG | YMHWYQKPG   | KAPKLLIYDT | SKLASGVPSR | 60  |
| Light chain    | FSGSGSGTEF | TLTISSLQPD | DFATYYCFQG | SGYPFTFGGG  | TKLEIKRTVA | APSVFIFPPS | 120 |
|                | DEQLKSGTAS | VVCLLNNFYP | REAKVQWKVD | NALQSGNSQE  | SVTEQDSKDS | TYSLSTLTTL | 180 |
|                | SKADYEKKHV | YACEVTHQGL | SSPVTKSFNR | GEC         |            |            |     |
|                | 213        |            |            |             |            |            |     |
| SEQ ID NO:38   | QVTLRESGPA | LVKPTQTTLT | TCTFSGFSLA | PTSSSTKKKTQ | LQLEHLLLDL | QMLNGINNY  | 60  |
| Light chain    | KNPKLTRMLT | AKFYMPKKAT | ELKHLQCLEE | ELKPLEEVLN  | LAQSKNFHLR | PRDLISINIV | 120 |
|                | IVLELKGSET | TFMCEYADET | ATIVEFLNRW | ITFCQSIIST  | LTSTSGMSVG | WIRQPPGKAL | 180 |
|                | EWLADIWDD  | KKDYNPSLKS | RLTISKDTSK | NQVVLKVTNM  | DPADTATYYC | ARSMITNWYF | 240 |
|                | DVWGAGTTVT | VSSASTKGPS | VFPLAPSSKS | TSGGTAALGC  | LVKDYFPEPV | TVSWNSGALT | 300 |
|                | SGVHTFPAVL | QSSGLYSLSS | VVTVPSSSLG | TQTYICNVNH  | KPSNTKVDKR | VEPKSCDKTH | 360 |
|                | TCPPCPAPEL | LGGPSVFLFP | PKPKDTLMIS | RTPEVTCVVV  | AVSHEDPEVK | FNWYVDGVEV | 420 |
|                | HNATKPREE  | QYNSTYRVVS | VLTVLHQDWL | NGKEYKCKVS  | NKALAAPIEK | TISKAKGQPR | 480 |
|                | EPQVYTLPPS | REEMTKNQVS | LTCLVKGFYP | SDIAVEWESN  | GQPENNYKTT | PPVLDSGGSF | 540 |
|                | FLYSKLTVDK | SRWQQGNVFS | CSVMHEALHN | HYTQKSLSL   | PGK        |            | 583 |
| SEQ ID NO:39   | DIQMTQSPST | LSASVGDRT  | ITCKAQLSVG | YMHWYQKPG   | KAPKLLIYDT | SKLASGVPSR | 60  |
| Light chain    | FSGSGSGTEF | TLTISSLQPD | DFATYYCFQG | SGYPFTFGGG  | TKLEIKRTVA | APSVFIFPPS | 120 |
|                | DEQLKSGTAS | VVCLLNNFYP | REAKVQWKVD | NALQSGNSQE  | SVTEQDSKDS | TYSLSTLTTL | 180 |
|                | SKADYEKKHV | YACEVTHQGL | SSPVTKSFNR | GEC         |            |            |     |
|                | 213        |            |            |             |            |            |     |

The term “IL-4” (also referred to herein as “IL4”) refers to the cytokine known as interleukin 4, which is produced by Th2 T cells and by eosinophils, basophils, and mast cells. IL-4 regulates the differentiation of naïve helper T cells (Th0 cells) to Th2 T cells. Steinke and Borish, *Respir. Res.* **2001**, 2, 66-70. Upon activation by IL-4, Th2 T cells subsequently produce additional IL-4 in a positive feedback loop. IL-4 also stimulates B cell proliferation and class II MHC expression, and induces class switching to IgE and IgG<sub>1</sub> 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).

[00201] 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).

**[00202]** 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  $\beta$  and  $\gamma$  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).

**[00203]** 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<sup>+</sup> 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:12).

**[00204]** 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.

**[00205]** 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.

**[00206]** 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.

**[00207]** 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.

**[00208]** 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/m<sup>2</sup>/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.

**[00209]** 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.

**[00210]** 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.

**[00211]** 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.

**[00212]** 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).

**[00213]** 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.

**[00214]** 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.

**[00215]** 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<sup>+</sup> cytotoxic T cells (lymphocytes), Th1 and Th17 CD4<sup>+</sup> T cells, natural killer cells, dendritic cells and M1 macrophages. TILs include both primary and secondary TILs. "Primary TILs" are those that are obtained from patient tissue samples as outlined herein (sometimes referred to as "freshly harvested"), and "secondary TILs" are any TIL cell populations that have been expanded or proliferated as discussed herein, including, but not limited to bulk TILs, expanded TILs ("REP TILs") as well as "reREP TILs" as discussed herein. reREP TILs can include for example second expansion TILs or second additional expansion TILs (such as, for example, those described in Step D of Figure 8, including TILs referred to as reREP TILs).

**[00216]** 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 $\gamma$ ) 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.

**[00217]** 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.

**[00218]** 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.

**[00219]** 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.

[00220] 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.

[00221] 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.”

[00222] 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<sub>H</sub>) 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.

**[00223]** 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.

**[00224]** 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.

[00225] 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  $F_d$  fragment consisting of the  $V_H$  and  $CH1$  domains; (iv) a  $F_v$  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  $F_v$  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  $F_v$  (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.

[00226] 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.

[00227] 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.

[00228] 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<sub>H</sub> and V<sub>L</sub> regions of the recombinant antibodies are sequences that, while derived from and related to human germline V<sub>H</sub> and V<sub>L</sub> sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[00229] As used herein, “isotype” refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by the heavy chain constant region genes.

[00230] 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.”

[00231] 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.

[00232] 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.

**[00233]** 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.

**[00234]** A “diabody” is a small antibody fragment with two antigen-binding sites. The fragments comprises a heavy chain variable domain (V<sub>H</sub>) connected to a light chain variable domain (V<sub>L</sub>) in the same polypeptide chain (V<sub>H</sub>-V<sub>L</sub> or V<sub>L</sub>-V<sub>H</sub>). 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.

**[00235]** 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<sup>-/-</sup> 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.



[00236] “Pegylation” refers to a modified antibody, or a fragment thereof, that typically is reacted with polyethylene glycol (PEG), such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. Pegylation may, for example, increase the biological (*e.g.*, serum) half life of the antibody. Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the term “polyethylene glycol” is intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C<sub>1</sub>-C<sub>10</sub>)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.

[00237] 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.

**[00238]** The term “recombinant lentiviral RNA molecule” refers to a single stranded RNA genome that may comprise at least a portion of a lentivirus genome, including 5’ and 3’ long terminal repeat (LTR) sequences. In some embodiments, the lentivirus genome may be modified to inhibit replication and limit pathogenicity, while retaining function. For example, the *env* gene, the *gag* gene, the *pol* gene, and the *rev* gene may be removed from the lentivirus genome that is comprised by the recombinant lentiviral RNA molecule. In some embodiments, the recombinant lentiviral RNA molecule is comprised by a lentiviral vector or a recombinant lentiviral particle as described elsewhere in this disclosure.

**[00239]** The term “lentiviral particle” or “lentiviral virion” refers to lentivirus, which is a subset of retrovirus. Lentiviruses can deliver significant amounts of genetic information into host cells and integrate it into the cellular genome, making genetically-engineered lentiviruses one of the most efficient tools of gene delivery. These lentiviruses contain a promoter which is used to control the expression of a transgene or shRNA but no virulence genes, making them safe to use in the laboratory.

**[00240]** The term “recombinant lentiviral particle” or “recombinant lentiviral virion” refers to a lentiviral particle or lentiviral virion produced by gene recombinant technologies. A recombinant lentiviral particle or lentiviral virion can be produced using any suitable method, such as by transducing or transfecting a packaging cell-line with a nucleic acid encoding the viral genome and subsequently isolating newly packaged viral particles. It is understood that

the recombinant technologies may be performed at a stage upstream of production of the viral vector itself. For example, recombinant technologies may be used to produce a plasmid, and the plasmid may then be produced at a larger scale, and finally the plasmid may be introduced into a cell line for packaging to produce the viral vector.

[00241] The term “lentiviral vector” refers to a recombinant lentiviral particle comprising a recombinant lentiviral RNA molecule, which contains at least a portion of a lentivirus genome, including 5' and 3' LTRs, and a nucleotide sequence encoding one or more genes of interest (GOIs). The lentivirus genome may be modified to inhibit replication and limit pathogenicity, while retaining function. For example, the *env* gene, the *gag* gene, the *pol* gene, and the *rev* gene may be removed from the lentivirus genome and included in helper plasmids.

[00242] The term “transfer vector” refers to a recombinant DNA plasmid containing a nucleotide sequence encoding the recombinant lentiviral RNA molecule, which contains at least a portion of a lentivirus genome, including 5' and 3' LTRs, and a nucleotide sequence encoding one or more genes of interest (GOIs), while one or more “helper plasmid(s)” or “envelope plasmid(s)” contain(s) genes for proteins that appear on the surface of the lentiviral particle or are essential for the function of the lentiviral particle. The transfer vector can be transfected into a packaging cell line along with the helper plasmid(s) to produce lentiviral vectors comprising the nucleotide sequence encoding the one or more GOIs as part of a recombinant lentiviral RNA molecule.

[00243] The term “Env” refers to the envelope glycoproteins on the surface of the lentiviral particle encoded by the *env* gene in the lentiviral genome. The Env protein contains a surface subunit and a transmembrane subunit. In some embodiments, the Env protein is selected from the group consisting of baboon retroviral envelope (Ba-EVTR), vesicular stomatitis-virus-G protein (VSV-G), and RD114.

[00244] The term “Gag” refers to the structural protein encoded by the *gag* gene in the lentiviral genome. The Gag protein is generated as a standalone protein or as a fusion protein with the Pol protein (Gag-Pol).

[00245] The term “Pol” refers to the reverse transcriptase and integrase enzymes encoded by the *pol* gene in the lentiviral genome. The Pol protein is generated as a fusion protein with the Gag protein (Gag-Pol).

[00246] The term “Rev” refers to the protein encoded by the *rev* gene in the lentiviral genome. Rev bears a leucine-rich nuclear export signal (NES) and, via association with the Rev response element (RRE), mediates nuclear-to-cytoplasmic transport of the partially spliced and unspliced RNAs, resulting in production of Gag, Gag-Pol, Env, and accessory proteins (Pollard & Malim, *Annu. Rev. Microbiol.*, **1998**, 52, 491532).

### III. Nucleic Acid Molecules Encoding Cytokines

[00247] Provided herein are nucleic acid molecules comprising a nucleotide sequence encoding one or more genes of interest (GOIs), e.g., cytokines selected from the group consisting of IL-12, IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNF $\alpha$ , IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof. In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12). In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a TeIL-12 and a tethered IL-15 (TeIL-15). In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a TeIL-12 and a tethered IL-2 (TeIL-2).

[00248] In some embodiments, the nucleotide sequences encoding the one or more GOIs, e.g., cytokines selected from the group consisting of IL-12, IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNF $\alpha$ , IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof, are provided by two or more nucleic acid molecules. For example, provided herein are a nucleic acid molecule comprising a nucleotide sequence encoding IL-12 or a variant thereof; and a second nucleic acid molecule comprising a nucleotide sequence encoding IL-15 or a variant thereof. In some embodiments, provided herein are a nucleic acid molecule comprising a nucleotide sequence encoding IL-12 or a variant thereof; and a second nucleic acid molecule comprising a nucleotide sequence encoding IL-2 or a variant thereof.

[00249] In some embodiments, the cytokine is a tethered cytokine. For example, the cytokine is linked to a cell membrane anchor moiety that allows the tethering of the cytokine to the cell surface. Suitable cell membrane anchor moieties include, for example, transmembrane domains of endogenous cell surface proteins and fragments thereof. Exemplary transmembrane domains that can be used include, for example, B7-1, B7-2, and CD8a transmembrane domains and fragments thereof. In some embodiments, the cell membrane anchor moiety further includes a transmembrane and intracellular domain of an

endogenous cell surface protein or fragment thereof. In some embodiments, the cell membrane anchor moiety is a B7-1, B7-2 or CD8a transmembrane-intracellular domain or fragment thereof. In certain embodiments, the cell membrane anchor moiety is a CD8a transmembrane domain having the amino acid sequence of IYIWAPLAGTCGVLLSLVIT (SEQ ID NO:40). In certain embodiments, the cell membrane anchor moiety is a B7-1 transmembrane-intracellular domain having the amino acid sequence of LLPSWAITLISVNGIFVICCLTYCFAPRCRERRRNERLRRESVRPV (SEQ ID NO:41).

**[00250]** In certain embodiments, the cell membrane anchor moiety is a non-peptide cell membrane anchor moiety. In exemplary embodiments, the non-peptide cell membrane anchor moiety is a glycosphosphatidylinositol (GPI) anchor. GPI anchors have a structure that includes a phosphoethanolamine linker, glycan core, and phospholipid tail. In some embodiments, the glycan core is modified with one or more side chains. In some embodiments, the glycan core is modified with one or more of the following side chains: a phosphoethanolamine group, mannose, galactose, sialic acid, or other sugars.

**[00251]** The membrane anchored cytokine may include linkers that allow for the linkage of components of the membrane anchored cytokine (e.g. a cytokine to a cell membrane anchor moiety). Suitable linkers include linkers that are at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acid residues in length. In some embodiments, the linker is 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 45-50, 50-60 amino acids in length. Suitable linkers include, but are not limited to: a cleavable linker, a non-cleavable linker, a peptide linker, a flexible linker, a rigid linker, a helical linker, or a non-helical linker. In some embodiments, the linker is a peptide linker that optionally comprises Gly and Ser. In certain embodiments, the peptide linker utilize a glycine-serine polymer, including for example (GS)<sub>n</sub> (SEQ ID NO:42), (GSGGS)<sub>n</sub> (SEQ ID NO:43), (GGGS)<sub>n</sub> (SEQ ID NO:44), (GGGGS)<sub>n</sub> (SEQ ID NO:45), (GGGGGS)<sub>n</sub> (SEQ ID NO:46), and (GGGGGGS)<sub>n</sub> (SEQ ID NO:47), where n is an integer of at least one (and generally from 3 to 10). Additional linkers that can be used with the present compositions and methods are described in U.S. Patent Publication Nos. US 2006/0074008, US 20050238649, and US 2006/0024317, each of which is incorporated by reference herein in its entirety, and particularly in pertinent parts related to linkers. In some embodiments, the peptide linker is SGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO:48).

**[00252]** In some embodiments, the linker is a cleavable linker. In exemplary embodiments, the cleavable linker allows for the release of the cytokine into the tumor

microenvironment. Cleavable linkers are also useful in embodiments, wherein two membrane anchored cytokines are co-expressed in the same cell. In exemplary embodiments, the linker is a self-cleaving 2A peptide. See, *e.g.*, Liu et al., Sci. Rep. 7(1):2193 (2017), which is incorporated by reference in relevant parts relating to 2A peptides. 2A peptides are viral oligopeptides that mediate cleavage of polypeptides during translation in eukaryotic cells. In some embodiments, the 2A peptide includes a C-terminus having the amino acid sequence GDVEXiNPGP (SEQ ID NO:49), wherein Xi is any naturally occurring amino acid residue. In certain embodiments, the 2A peptide is a porcine teschovirus-1 2A peptide (GSGATNFSLLKQAGDVEENPGP, SEQ ID NO:50). In some embodiments, the 2A peptide is an equine rhinitis A virus 2A peptide (GSGQCTNYALLKLAGDVESNPGP, SEQ ID NO:51). In certain embodiments, the 2A peptide is a foot-and-mouth disease virus 2A peptide: (GSGEGRGSLLTCGDVEENPGP, SEQ ID NO:52). In some embodiments, the cleavable linker includes a furin-cleavable sequence. Exemplary furin-cleavable sequences are described for example, Duckert et al., Protein Engineering, Design & Selection 17(1):107-112 (2004), and US Patent No. 8,871,906, each of which is incorporated herein by reference, particularly in relevant parts relating to furin-cleavable sequences. In some embodiments, the linker includes a 2A peptide and a furin-cleavable sequence. In exemplary embodiments, the furin-cleavable 2A peptide includes the amino acid sequence RAKRSGSGATNFSLLKQAGDVEENPGP (SEQ ID NO:53).

**[00253]** In some embodiments, the linker is a degradable linker (*e.g.*, a disulfide linker) such that under physiological conditions, the linker degrades, thereby releasing the cytokine. In some embodiments, the cytokine is reversibly linked to functional groups through a degradable linker such that under physiological conditions, the linker degrades and releases the cytokine. Suitable degradable linkers include, but are not limited to: a protease sensitive linker that is sensitive to one or more enzymes present in biological media such as proteases in a tumor microenvironment such a matrix metalloproteases present in a tumor microenvironment or in inflamed tissue (*e.g.* matrix metalloproteinase 2 (MMP2) or matrix metalloproteinase 9 (MMP9)).

**[00254]** In other embodiments, the linker is an enzyme-sensitive linker. Exemplary cleavable linker include those that are recognized by one of the following enzymes: metalloprotease MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-14, plasmin, PSA, PSMA, CATHEPSIN D, CATHEPSIN K, CATHEPSIN S, ADAM10, ADAM12, ADAMTS, Caspase-1, Caspase-2, Caspase-3, Caspase-4, Caspase-5, Caspase-6, Caspase-7, Caspase-8,

Caspase-9, Caspase-10, Caspase-11, Caspase-12, Caspase-13, Caspase-14, and TACE. See, e.g., US Patent Nos. 8,541,203 and 8,580,244, each of which is incorporated by reference in its entirety and in pertinent parts related to cleavable linkers.

**[00255]** In certain embodiments, the membrane anchored cytokine includes a signal peptide that facilitates the translocation of the cytokine to the cell membrane. Any suitable signal peptide that facilitates the localization of the cytokine to the cell membrane can be used. In some embodiments, the signal peptide does not interfere with the bioactivity of the cytokine. Exemplary signal peptide sequences include, but are not limited to: human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor signal sequence, human prolactin signal sequence, and human IgE signal sequence. In certain embodiments, the fusion protein includes a human IgE signal sequence. In exemplary embodiments, the human IgE signal sequence has the amino acid sequence MDWTWILFLVAAATRVHS (SEQ ID NO:54). In some embodiments, the human IgE signal sequence includes the amino acid sequence NIKGSPWKGSLLLLLVSNLLLCQSVAP (SEQ ID NO:55). In some embodiments, the signal peptide sequence is an IL-2 signal sequence having the amino acid sequence MYRMQLLSIALSLALVTNS (SEQ ID NO:56).

**[00256]** The nucleic acid molecule can further comprise a native or normative promoter operably linked to the nucleotide sequence encoding one or more cytokines. In some embodiments, the nucleotide sequence encoding each cytokine is operably linked to the same promoter. In some embodiments, the nucleotide sequence encoding each cytokine is operably linked to a different promoter. Preferably, the promoter is functional in T cells. The selection of a promoter, e.g., strong, weak, inducible, tissue-specific and developmental-specific, is within the ordinary skill of the artisan. Similarly, the combining of a nucleotide sequence with a promoter is also within the skill of the artisan. The promoter can be a non-viral promoter or a viral promoter, e.g., a nuclear factor of activated T-cells (NFAT) promoter, an EF-1a promoter, a cytomegalovirus (CMV) promoter, a CAG promoter, an MND promoter, or an SSFV promoter, an SV40 promoter, an RSV promoter, or a promoter found in the long-terminal repeat of the murine stem cell virus.

**[00257]** “NFAT promoter” as used herein means one or more NFAT responsive elements linked to a minimal promoter of any gene expressed by T-cells. Preferably, the minimal promoter of a gene expressed by T-cells is a minimal human IL-2 promoter. The NFAT responsive elements may comprise, e.g., NFAT1, NFAT2, NFAT3, and/or NFAT4 responsive elements. The NFAT promoter (or functional portion or functional variant



thereof) may comprise any number of binding motifs, e.g., at least two, at least three, at least four, at least five, or at least six, at least seven, at least eight, at least nine, at least ten, at least eleven, or up to twelve binding motifs.

TABLE 4 – NFAT Promoter Related Sequences.

| Description                   | Amino Acid Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|-------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 6X NFAT IL-2 minimal promoter | TCGAGGTCGACGGTATCGATAAGCTTGAT<br>ATCGAATTAGGAGGAAAACTGTTTCATA<br>CAGAAGGCGTCAATTAGGAGGAAAACTG<br>TTTCATACAGAAGGCGTCAATTAGGAGGA<br>AAAACGTGTTTCATACAGAAGGCGTCAATT<br>GGTCCCATCGAATTAGGAGGAAAACTGT<br>TTCATACAGAAGGCGTCAATTAGGAGGAA<br>AAACTGTGTTTCATACAGAAGGCGTCAATTA<br>GGAGGAAAACTGTTTCATACAGAAGGCG<br>TCAATTGGTCCCGGGACATTTTGACACCCC<br>CATAATATTTTTCCAGAATTAACAGTATAA<br>ATTGCATCTCTTGTTCAAGAGTTCCTATC<br>ACTCTCTTAATCACTACTCACAGTAACCT<br>CAACTCCTGGCCACC (SEQ ID NO: 57) |
| NFAT responsive element       | GGAGGAAAACTGTTTCATACAGAAGGCG<br>T (SEQ ID NO: 58)                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Human IL-2 Promoter           | CATTTTGACACCCCCATAATATTTTCCAG<br>AATTAACAGTATAAATTGCATCTCTTGTC<br>AAGAGTTCCTATCACTCTCTTAATCACT<br>ACTCACAGTAACCTCAACTCTG (SEQ ID<br>NO:59)                                                                                                                                                                                                                                                                                                                                    |

**[00258]** In a preferred embodiment, the NFAT promoter comprises six NFAT binding motifs. See, e.g., US Patent No. 8,556,882, which is incorporated by reference in its entirety and particularly for pertinent parts relating to NFAT promoters. In some embodiments, the NFAT promoter system controls expression of one or more cytokines. In certain embodiments, the cytokine is selected from the group consisting of IL-12, IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof. In some embodiments, the cytokine is a tethered cytokine. In some embodiments, the NFAT promoter system controls expression of IL-12, or a variant thereof. In some embodiments, the NFAT promoter system controls expression of IL-15, or a variant thereof. In some embodiments, the NFAT promoter system controls expression of IL-18, or a variant thereof. In some embodiments, the NFAT promoter system controls expression of TeIL-12. In some embodiments, the NFAT promoter system controls

expression of TeIL-15. In some embodiments, the NFAT promoter system controls expression of TeIL-18.

**[00259]** As used herein, “interleukin 12”, “IL-12” and “IL12” all refer to an interleukin that is a heterodimeric cytokine encoded by the IL-12A and IL-12B genes (Genbank Accession numbers: NM\_000882 (IL-12A) and NM\_002187 (IL-12B)). IL-12 is composed of a bundle of four alpha helices and is involved in the differentiation of native T cells into TH1 cells. It is encoded by two separate genes, IL-12A (p35) and IL-12B (p40). The active heterodimer (referred to as 'p70'), and a homodimer of p40 are formed following protein synthesis. IL-12 binds to the IL-12 receptor, which is a heterodimeric receptor formed by IL-12R-β1 and IL-12R-β2. IL-12 is known as a T cell-stimulating factor that can stimulate the growth and function of T cells. In particular, IL-12 can stimulate the production of interferon gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells and reduce IL-4 mediated suppression of IFN-γ. IL-12 can further mediate enhancement of the cytotoxic activity of NK cells and CD8+ cytotoxic T lymphocytes. Moreover, IL-12 can also have anti-angiogenic activity by increasing production of interferon gamma, which in turn increases the production of the chemokine inducible protein-10 (IP-10 or CXCL10). IP-10 then mediates this anti-angiogenic effect. Thus, without being bound by any particular theory of operation, it is believed that IL-12 can increase the survivability and/or anti-tumor effects of the TIL compositions provided herein.

**[00260]** In some embodiments, the IL-12 is a full length IL-12, a fragment or a variant of IL-12. In some embodiments, the IL-12 is a human IL-12 or a variant human IL-12. In exemplary embodiments, the IL-12 is a biological active human IL-12 variant. In some embodiments, the IL-12 includes a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations as compared to a wild-type IL-12.

**[00261]** In some embodiments, the IL-12 comprises an IL-12 p35 subunit or a variant thereof. In some embodiments, the IL-12 p35 subunit is a human IL-12 p35 subunit. In some embodiments, the IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60. In certain embodiments, the IL-12 comprises an IL-12 p40 subunit or a variant thereof. In some embodiments, the IL-12 p40 subunit has the amino acid sequence of SEQ ID NO:61. In certain embodiments, the IL-12 is a single chain IL-12 polypeptide comprising an IL-12 p35 subunit attached to an IL-12 p40 subunit. Such IL-12 single chain polypeptides advantageously retain one or more of the biological activities of wildtype IL-12. In some embodiments, the single chain IL-12 polypeptide described herein is according to the

formula, from N-terminus to C-terminus, (p40)-(L)-(p35), wherein “p40” is an IL-12 p40 subunit, “p35” is IL-12 p35 subunit and L is a linker. In other embodiments, the single chain IL-12 is according to the formula from N-terminus to C-terminus, (p35)-(L)-(p40). Any suitable linker can be used in the single chain IL-12 polypeptide including those described herein. Suitable linkers can include, for example, linkers having the amino acid sequence (GGGGS)<sub>x</sub> wherein x is an integer from 1-10. Other suitable linkers include, for example, the amino acid sequence GGGGGGS. Exemplary single chain IL-12 linkers that can be used with the subject single chain IL-12 polypeptides are also described in Lieschke et al., *Nature Biotechnology* 15: 35-40 (1997), which is incorporated herein in its entirety by reference and particularly for its teaching of IL-12 polypeptide linkers. In an exemplary embodiment, the single chain IL-12 polypeptide is a single chain human IL-12 polypeptide (*i.e.*, it includes a human p35 and p40 IL-12 subunit).

TABLE 5 – IL-12 Related Sequences.

| Description                  | Amino Acid Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-12 p35 subunit      | RNLPVATPDPGMFPCLLHHSQNLLRAVSNML<br>QKARQTLEFYPTCTSEEIDHEDITKDKTSTVEA<br>CLPLELTKNESCLNSRETSFITNGSCLASRKT<br>SFMALCLSSIYEDLKMYQVEFKTMNAKLL<br>MDPKRQIFLDQNMLAVIDELMQALNFNSET<br>VPQKSSLEEDFYKTKIKLCILLHAFRIRAVTI<br>DRVMSYLNAS (SEQ ID NO:60)                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| Human IL-12 p40 subunit      | IWELKKDVYVVELDWYPDAPGEMVVLTCDD<br>TPEEDGITWTLDSQSSEVLGSGKTLTIQVKEF<br>GDAGQYTCHKGGVLSHSLLLHKKEDGIW<br>STDILKDQKEPKNTFLRCEAKNYSGRFTC<br>WWLTITISTDLTFSVKSSRGSSDPQGVTCGAA<br>TLAERVRGDNKEYEYSVEQCQEDSACPAAE<br>ESLPIEVMVDAVHKLKYENYTSSFFIRDIIKP<br>DPPKNLQLKPLKNSRQVEVSWEYPDTWSTP<br>HSYFSLTFCVQVQGKSKREKKDRVFTDKTS<br>ATVICRKNASISVRAQDRYYSSSWSEWASVP<br>CS (SEQ ID NO:61)                                                                                                                                                                                                                                                                                                                                              |
| TeIL-12-Lr1-Ar2 (amino acid) | MDWTWILFLVAAATRVIIISIWELKKDVYVV<br>ELDWYPDAPGEMVVLTCDDTPEEDGITWTL<br>DSQSSEVLGSGKTLTIQVKEFGDAGQYTCHKG<br>GEVLSHSLLLHKKEDGIWSTDILKDQKEPK<br>NTFLRCEAKNYSGRFTCWWLTTISTDLTFS<br>VKSSRGSSDPQGVTCGAATLSAERVRGDNK<br>EYYSVEQCQEDSACPAAEESLPIEVMVDAVH<br>KLKYENYTSSFFIRDIIKPDPPKNLQLKPLKN<br>SRQVEVSWEYPDTWSTPHSYFSLTFCVQVQ<br>GKSKREKKDRVFTDKTSATVICRKNASISVR<br>AQDRYYSSSWSEWASVPCSGGGGGGSRNLP<br>VATPDPGMFPCLLHHSQNLLRAVSNMLQKAR<br>QTLEFYPTCTSEEIDHEDITKDKTSTVEACLPL<br>ELTKNESCLNSRETSFITNGSCLASRKTSM<br>MALCLSSIYEDLKMYQVEFKTMNAKLLMDP<br>KRQIFLDQNMLAVIDELMQALNFNSETVPQ<br>KSSLEEDFYKTKIKLCILLHAFRIRAVTIDRV<br>MSYLNASSGGGGSGGGGSGGGGSGGGGSG<br>GGSLQLLPSWAITLISVNGIFVICCLTYCFAPR<br>CRERRRNERLRRESVRPV (SEQ ID NO:62) |

|                              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| TeIL-12-Lr1-Ar2 (nucleotide) | ATGGATTGGACCTGGATTCTGTTCTCGTGGCCGC<br>CGCTACCCGCGTGCACTCCATCTGGGAGCTGAAG<br>AAAGACGTGTACGTGGTGAATTGGATTGGTACC<br>CCGACGCCCCAGGCGAGATGGTGGTTCTGACTTG<br>CGACACGCCGGAGGAGGACGGCATCACCTGGAC<br>GCTGGACCAGAGCAGCGAAGTGCTGGGCTCTGG<br>TAAGACCTTAACCATCCAGGTCAAGGAGTTCGGT<br>GATGCAGGACAGTACACGTGTCAACAAGGGCGGG<br>GAGGTCTTGTCCCATAGCCTGCTGCTCCTGCACAA<br>GAAGGAAGACGGCATCTGGTCTACGGATATCCTT<br>AAAGACCAGAAGGAGCCCAAGAACAAAACCTTCC<br>TGCGATGCGAGGCTAAGAATTACTCCGGGCGCTT<br>TACCTGTTGGTGGCTCACAACCATCTCGACCGACC<br>TGACCTTCTCCGTGAAGTCTTCAGGGGATCTTCT<br>GATCCGCAAGGCGTTACCTGCGGGGCCGCTACCC<br>TGTCGGCTGAGCGCGTCCGTGGTGACAACAAGGA<br>GTACGAGTACTCGGTGGAATGCCAGGAGGACAG<br>CGCCTGCCAGCGGCCGAAGAGTCCTTGCCCATC<br>GAGGTGATGGTGGACGCTGTACACAAGCTCAAGT<br>ACGAGAACTACACCTCCTCCTTTATACGCGAC<br>ATCATCAAGCCTGACCCCCCTAAAAACCTGCAGCT<br>GAAGCCCCCTAAAAACTCTCGACAGGTCGAGGTA<br>TCCTGGGAGTACCCCGACACCTGGTCTACTCCTCA<br>TTCGTATTTAGCCTGACCTTCTGCGTGCAGGTGC<br>AGGGCAAGTCCAAGCGCGAGAAGAAGGACCGCG<br>TGTTACCGACAAAACAGCGCGACTGTCATTTGT<br>CGGAAGAACGCGAGCATCTCCGTACGGGCACAG<br>GATCGCTATTACTCCAGCAGTTGGTCAGAGTGGG<br>CTTCGGTTCCGTGTTCTGGAGGAGGTGGCGGCGG<br>CTCACGCAACCTGCCCGTGGCGACGCCGACCCG<br>GGTATGTTTCCTTGTCTGCACCACTCTCAGAACTT<br>GCTCCGTGCCGTCTCGAATATGCTGCAGAAGGCC<br>CGCCAGACTCTGGAGTTCTATCCATGCACTAGTGA<br>GGAGATCGATCATGAGGACATCACCAAGGACAA<br>GACATCCACCGTGGAGGCTTGTCTGCCGCTGGAG<br>TTGACAAAGAACGAGAGCTGCCTCAATAGCCGCG<br>AGACTTCCTTCATACCAACGGCTCCTGCCTCGCG<br>AGCCGTAAGACCTCCTTTATGATGGCGCTGTGCCT<br>GTCAAGTATCTACGAAGATCTGAAAATGTATCAG<br>GTGGAGTTCAAACTATGAACGCAAACTCCTGA<br>TGGATCCCAAGCGCAAATTTCTTGGACCAGAAC<br>ATGCTTGCCGTGATCGACGAGCTGATGCAGGCC<br>TGAACCTCAACTCCGAGACCGTCCCTCAGAAGTCG<br>TCTCTGGAGGAACCCGACTTTTACAAGACAAAGA<br>TCAAGCTGTGCATCCTGCTACACGCGTTCCGCATT<br>CGGGCCGTGACCATTGACCGCGTGATGTCCTACC<br>TGAATGCCTCGTCCGGCGGGGGGGCTCCGGTG<br>GGGGCGGCTCTGGCGGAGGCGGCTCAGGTGGCG<br>GCGGCTCCGGCGGAGGTTGCTTCAGCTTCTGCC<br>CAGTTGGGCAATCACTCTGATTCCGTCAACGGAA<br>TCTTCGTCTCTGCTGCCTGACCTACTGCTTGTCTC |
|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

|  |                                                                                      |
|--|--------------------------------------------------------------------------------------|
|  | CCAGATGTAGAGAGCGCCGCCGCAACGAGCGCCT<br>GCGTCGTGAGAGCGTGAGGCCTGTGTGA {SEQ<br>ID NO:63} |
|--|--------------------------------------------------------------------------------------|

**[00262]** In exemplary embodiments the nucleic acid molecule disclosed herein encodes a TeIL-12, comprising the amino acid sequence of SEQ ID NO: 62 (amino acids 1-18: human IgE signal sequence peptide; amino acids 529-553: peptide linker; amino acids 554-601: membrane anchor), wherein the nucleic acid is operably linked to an NFAT promoter, an EF-1a promoter, a CMV promoter, a CAG promoter, an MND promoter, or an SSFV promoter, as described herein. See, e.g., US Patent No. 8,556,882, which is incorporated by reference in its entirety and particularly for pertinent parts relating to NFAT promoters for IL-12 expression.

**[00263]** In some embodiments, the nucleic acid molecule further comprises a nucleotide sequence encoding a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof. In some embodiments, the cytokine is a tethered cytokine. In some embodiments, the cytokine is under the control of an EF1a promoter, a CMV promoter, a CAG promoter, an MND promoter, or an SSFV promoter.

**[00264]** As used herein, “interleukin 15”, “IL-15” and “IL15” all refer to an interleukin that binds to and signals through a complex composed of an IL-15 specific receptor alpha chain (IL-15R $\alpha$ ), an IL-2/IL-15 receptor beta chain (CD122) and the common gamma chain (gamma-C, CD132) (e.g., Genbank Accession numbers: NM\_00000585, NP\_000576 and NP\_751915 (human); and NM\_001254747 and NP\_001241676 (mouse)). IL-15 has been shown to stimulate T cell proliferation inside tumors. IL-15 also is able to extend the survivability of effector memory CD8<sup>+</sup> T cells and is critical for the development of NK cells. Therefore, without being bound by any particular theory of operation, it is believed that modified TILs associated with an IL-15s described herein exhibit enhanced survival and/or anti-tumor effects.

**[00265]** IL-15 has a short half-life of less than 40 minutes in vivo. Modifications to IL-15 monomer can improve its in vivo pharmacokinetics in the treatment of cancers. These modifications have generally centered on improving the trans-presentation of IL-15 with the alpha subunit of IL-15 receptor, IL-15R $\alpha$ . Such modifications include: 1) pre-association of IL-15 and its soluble receptor  $\alpha$ -subunit-Fc fusion to form IL-15: IL-15R $\alpha$ -Fc complex (see, e.g., Rubinstein et al., Proc Natl Acad Sci U.S.A. 103:9166–71 (2006)); 2) expression of the

superagonist IL-15-sIL-15R $\alpha$ -sushi protein (see, *e.g.*, Bessard et al., Molecular cancer therapeutics 8: 2736-45 (2009)); and 3) pre-association of human IL-15 mutant IL-15N72D with IL-15R $\alpha$ -Fc sushi-Fc fusion complex (see, *e.g.*, Zhu et al., Journal of Immunology 183: 3598-6007 (2009)).

[00266] In some embodiments, the IL-15 is a tethered IL-15 (TeIL-15). In some embodiments, the TeIL-15 comprises the amino acid sequence of SEQ ID NO:73 (amino acids 1-18: human IgE signal sequence peptide; amino acids 132-157: peptide linker; amino acids 158-205: membrane anchor).

[00267] In some embodiments, the IL-15 is a full length IL-15, a fragment or a variant of IL-15. In some embodiments, the IL-15 is a human IL-15 or a variant human IL-15. In exemplary embodiments, the IL-15 is a biological active human IL-15 variant. In some embodiments, the IL-15 includes a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations as compared to a wild-type IL-15. In certain embodiments, the IL-15 includes an N72D mutation relative to a wild type human IL-15. In some embodiments, the variant IL-15 exhibits IL-15R $\alpha$  binding activity.

[00268] In some embodiments, the IL-15 includes an IL-15 and an extracellular domain of an IL-15R $\alpha$ . In certain embodiments, the IL-15 includes an IL-15 and an IL-15R $\alpha$  fused to an Fc domain (IL-15R $\alpha$ -Fc)

TABLE 6 – IL-15 Related Sequences.

| Description                     | Amino Acid Sequence                                                                                                                                                                                                                                                                                                                                           |
|---------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-15 (N72D mutant)       | NWVNVISDLKKIEDLIQSMHIDATLYTESDV<br>HPSCKVTAMKCFLELQVISLESGDASHDT<br>VENLILANDSLSSNGNVTESGCKECELEEK<br>NIKEFLQSFVHIVQMFINTS (SEQ ID NO: 64)                                                                                                                                                                                                                    |
| Human IL-15R-alpha-Su/Fc domain | ITCPPPMSVEHADIWVKSYSLYSRERYICNSG<br>FKRKAGTSSLTECVLNKATNVAHWTTPSLK<br>CIREPKSCDKTHTCPPCPAPELLGGPSVFLFP<br>PKPKDITLMISRTPEVTCVVVDVSHEDPEVKF<br>NWYVDGVEVHNAKTKPREEQYNSTYRVVS<br>VLTVLHQDWLNGKEYKCKVSNKALPAPIEK<br>TISKAKGQPREPQVYTLPPSRDELTKNQVSL<br>TCLVKGFYPSDIAVEWESNGQPENNYKTTTPP<br>VLDSDGSEFLYSKLTVDKSRWQQGNVFS<br>VMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 65) |



|                                                             |                                                                                                                                                                                                                                                                                                                           |
|-------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-15R-alpha-Su (65aa truncated extracellular domain) | ITCPPPMSEVEIADIWVKSYSLSYRERYICNSG<br>FKRKAGTSSLTECVLNKATNVAHWTTPSLK<br>CIR (SEQ ID NO: 66)                                                                                                                                                                                                                                |
| Human IL-15 isoform 2                                       | MVLGTIDLCSFCSAGLPKTEANWVNVISDLK<br>KIEDLIQSMHIDATLYTESDVHPSCKVTAMK<br>CFLELQVISLESGDASIHDTVENLILANNSL<br>SSNGNVTESGCKECELEEKNIKEFLQSFVHI<br>VQMFINTS (SEQ ID NO: 67)                                                                                                                                                      |
| Human IL-15 isoform 1                                       | MRISKPHLRSISIQCYLCLLLNSHFLTEAGIHV<br>FILGCFCSAGLPKTEANWVNVISDLKKIEDLIQ<br>SMHIDATLYTESDVHPSCKVTAMKCFLELQ<br>QVISLESGDASIHDTVENLILANNSLSSNGN<br>VTESGCKECELEEKNIKEFLQSFVHIVQMF<br>INTS (SEQ ID NO: 68)                                                                                                                     |
| Human IL-15 (without signal peptide)                        | NWVNVISDLKKIEDLIQSMHIDATLYTESDV<br>HPSCKVTAMKCFLELQVISLESGDASIHDT<br>VENLILANNSLSSNGNVTESGCKECELEE<br>KNIKEFLQSFVHIVQMFINTS (SEQ ID NO: 69)                                                                                                                                                                               |
| Human IL-15R-alpha (85 aa truncated extracellular domain)   | ITCPPPMSEVHADIWVKSYSLSYRERYICNSG<br>FKRKAGTSSLTECVLNKATNVAHWTTPSLK<br>CIRDPALVHQRPAPPSTVTTAGV (SEQ ID<br>NO: 70)                                                                                                                                                                                                          |
| Human IL-15R-alpha (182aa truncated extracellular domain)   | ITCPPPMSEVHADIWVKSYSLSYRERYICNSG<br>FKRKAGTSSLTECVLNKATNVAHWTTPSLK<br>CIRDPALVHQRPAPPSTVTTAGVTPQPESLSP<br>SGKEPAASSPSSNNTAATTAAIVPGSQLMPS<br>KSPSTGTTEISSHESHSHTPSQTTAKNWELTA<br>SASHQPPGVYPQGHSDTTVAISTST (SEQ ID<br>NO: 71)                                                                                             |
| Human IL-15R-alpha                                          | MAPRRARGCRTLGLPALLLLLLLRPPATRGI<br>TCPPPMSEVHADIWVKSYSLSYRERYICNSG<br>FKRKAGTSSLTECVLNKATNVAHWTTPSLK<br>CIRDPALVHQRPAPPSTVTTAGVTPQPESLSP<br>SGKEPAASSPSSNNTAATTAAIVPGSQLMPS<br>KSPSTGTTEISSHESHSHTPSQTTAKNWELTA<br>SASHQPPGVYPQGHSDTTVAISTSTVLLCGL<br>SAVSLACYLKSRQTPPLASVEMEAMEALP<br>VTWGTSSRDELENCSHHL (SEQ ID NO: 72) |
| Tethered IL-15 (amino acid)                                 | MDWTWILFLVAAATRVHSNWNVISDLKKIEDLIQ<br>SMHIDATLYTESDVHPSCKVTAMKCFLELQVISLES<br>GDASIHDTVENLILANNSLSSNGNVTESGCKECEEL<br>EEKNIKEFLQSFVHIVQMFINTSSGGGGSGGGSGG<br>GGSGGGSGGGSLQLPSWAITLISVNGIFVICCLTY<br>CFAPRCRERRRNERLRRESVRPV (SEQ ID NO: 73)                                                                               |
| Tethered IL-15 (nucleotide)                                 | ATGGATTGGACCTGGATCCTGTTCTGGTGGCTG<br>CGGCCACCGTGACACAGCAACTGGGTCAATGT<br>GATCTCCGACCTGAAGAAGATTGAGGACCTCATC                                                                                                                                                                                                               |

|  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|--|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|  | CAGTCTATGCACATTGACGCAACACTTTACACCGA<br>GAGCGATGTGCACCCCTCTTGCAAGGTGACTGCC<br>ATGAAATGTTTCTTGCTCGAATTGCAGGTCATCTC<br>TCTGGAGAGTGGGGACGCTTCCATACATGACACC<br>GTGGAGAACCTGATCATCTGGCCAACAACAGCC<br>TCTCGTCAAATGGCAACGTGACTGAGAGCGGGTG<br>CAAAGAGTGTGAGGAGCTGGAGGAGAAGAACAT<br>CAAGGAGTTCCTGCAGAGCTTCGTCCACATCGTG<br>CAGATGTTTATTAACACGTCGTCCGGAGGCGGGG<br>GCTCTGGTGGCGCGGCTCCGGGGGAGGCGGCT<br>CCGGTGGCGGTGGTTCCGGCGGAGGCTCGCTAC<br>AGCTGCTGCCAGTTGGGCCATCACCTTATTTCT<br>GTCAACGGCATCTTCGTTATCTGTTGCCTGACCTA<br>CTGCTTTGCGCCGAGGTGTCGCGAGCGGCGTCGC<br>AACGAGCGCCTGCGCCGAGAATCCGTGCGCCCTG<br>TGTGA (SEQ ID NO:74) |
|--|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

[00269] In some embodiments the cytokine is a superagonist IL-15 (IL-15SA) that includes a complex of human IL-15 and soluble human IL-15R $\alpha$ . The combination of human IL-15 with soluble human IL-15R $\alpha$  forms an IL-15 SA complex that possesses greater biological activity than human IL-15 alone. Soluble human IL-15R $\alpha$ , as well as truncated versions of the extracellular domain, has been described in the art (Wei et al., 2001 J of Immunol. 167: 277-282). The amino acid sequence of human IL-15R $\alpha$  is set forth in SEQ ID NO: 72. In some embodiments, the IL-15SA includes a complex of human IL-15 and soluble human IL-15R $\alpha$  comprising all or a portion of the extracellular domain, without the transmembrane or cytoplasmic domain. In some embodiments, the IL-15SA includes a complex of human IL-15 and soluble human IL-15R $\alpha$  that includes the full extracellular domain or a truncated form of the extracellular domain which retains IL-15 binding activity.

[00270] In some embodiments, the IL-15SA includes a complex of human IL-15 and soluble human IL-15R $\alpha$  that includes a truncated form of the extracellular domain which retains IL-15 binding activity. In some embodiments, the soluble human IL-15R $\alpha$  includes amino acids 1-60, 1-61, 1-62, 1-63, 1-64 or 1-65 of human IL-15R $\alpha$ . In some embodiments, the soluble human IL-15R $\alpha$  includes amino acids 1-80, 1-81, 1-82, 1-83, 1-84 or 1-85 of human IL-15R $\alpha$ . In some embodiments, the soluble human IL-15R $\alpha$  includes amino acids 1-180, 1-181, or 1-182 of human IL-15R $\alpha$ .

[00271] In some embodiments, the cytokine is an IL-15SA comprising a complex of human IL-15 and soluble human IL-15R $\alpha$  comprising a truncated form of the extracellular domain which retains IL-15 binding activity and comprises a Sushi domain. The Sushi domain of IL-15R $\alpha$  is described in the art as approximately 60 amino acids in length and

comprises 4 cysteines. (Wei et al., 2001). Truncated forms of soluble human IL-15R $\alpha$  which retain IL-15 activity and comprise a Sushi domain are useful in IL-15SA of the present disclosure.

**[00272]** In some embodiments, the cytokine includes a complex comprising soluble human IL-15R $\alpha$  expressed as a fusion protein, such as an Fc fusion as described herein (*e.g.*, human IgG1 Fc), with IL-15. In some embodiments, IL-15SA comprises a dimeric human IL-15R $\alpha$ Fc fusion protein (*e.g.*, human IgG1 Fc) complexed with two human IL-15 molecules.

**[00273]** In some embodiments, the cytokine is an IL-15SA cytokine complex that includes an IL-15 molecule comprising an amino acid sequence set forth in SEQ ID NO: 64, SEQ ID NO: 67, SEQ ID NO:68, or SEQ ID NO:69. In some embodiments, an IL-15SA cytokine complex comprises a soluble IL-15R $\alpha$  molecule comprising a sequence of SEQ ID NO:66, SEQ ID NO: 70 or SEQ ID NO:71.

**[00274]** In some embodiments, the cytokine is an IL-15SA cytokine complex that includes a dimeric IL-15R $\alpha$ Fc fusion protein complexed with two IL-15 molecules. In some embodiments, IL-15-SA comprises a dimeric IL-15R $\alpha$ Su (Sushi domain)/Fc (SEQ ID NO:65) and two IL-15N72D (SEQ ID NO:64) molecules (also known as ALT-803), as described in US20140134128, incorporated herein by reference. In some embodiments, the IL-15SA comprises a dimeric IL-15R $\alpha$ Su/Fc molecule (SEQ ID NO: 65) and two IL-15 molecules (SEQ ID NO: 67). In some embodiments, the IL-15SA comprises a dimeric IL-15R $\alpha$ Su/Fc molecule (SEQ ID NO: 65) and two IL-15 molecules (SEQ ID NO:68). In some embodiments, the IL-15SA comprises a dimeric IL-15R $\alpha$ Su/Fc molecule (SEQ ID NO:65) and two IL-15 molecules (SEQ ID NO:69).

**[00275]** In some embodiments, the IL-15SA includes a dimeric IL-15R $\alpha$ Su/Fc molecule (SEQ ID NO:65) and two IL-15 molecules having amino acid sequences selected from SEQ ID NO: 64, 67, 68, and 69.

**[00276]** In some embodiments, the IL-15SA includes a soluble IL-15R $\alpha$  molecule (SEQ ID NO:66) and two IL-15 molecules (SEQ ID NO:64). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO:66) and two IL-15 molecules (SEQ ID NO:67). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO:66) and two IL-15 molecules (SEQ ID NO:68). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO:66) and two IL-15 molecules (SEQ ID NO:69).

**[00277]** In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO:70) and two IL-15 molecules (SEQ ID NO:64). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO: 70) and two IL-15 molecules (SEQ ID NO:67). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO: 70) and two IL-15 molecules (SEQ ID NO:68). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO: 70) and two IL-15 molecules (SEQ ID NO:69).

**[00278]** In some embodiments, the IL-15SA includes a soluble IL-15R $\alpha$  molecule (SEQ ID NO:71) and two IL-15 molecules (SEQ ID NO:64). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO: 71) and two IL-15 molecules (SEQ ID NO:67). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO:71) and two IL-15 molecules (SEQ ID NO:68). In some embodiments, the IL-15SA comprises a soluble IL-15R $\alpha$  molecule (SEQ ID NO:71) and two IL-15 molecules (SEQ ID NO:69).

**[00279]** In some embodiments, the IL-15SA comprises a dimeric IL-15R $\alpha$ Su/Fc (SEQ ID NO:65) molecule and two IL-15 molecules (SEQ ID NO:68). In some embodiments, the IL-15SA includes a dimeric IL-15R $\alpha$ Su/Fc (SEQ ID NO:65) molecule and two IL-15 molecules (SEQ ID NO:69).

**[00280]** In some embodiments, the IL-15SA includes SEQ ID NO:65 and SEQ ID NO:66. In some embodiments IL-15SA comprises SEQ ID NO:67 or SEQ ID NO:68. In some embodiments the IL-15SA comprises SEQ ID NO:67 and SEQ ID NO:65. In some embodiments the IL-15SA comprises SEQ ID NO:68 and SEQ ID NO:65. In some embodiments the IL-15SA comprises SEQ ID NO:69 and SEQ ID NO:65. In some embodiments, the IL-15SA comprises SEQ ID NO:67 and SEQ ID NO:66. In some embodiments the IL-15SA comprises SEQ ID NO:68 and SEQ ID NO:66.

**[00281]** As used herein, “interleukin 18”, “IL-18,” “IL18,” “IGIF,” “IL-1g,” “interferon-gamma inducing factor,” and “IL1F4,” all refer to an interleukin that is a heterodimeric cytokine encoded by the IL-18 gene (e.g., Genbank Accession numbers: NM\_001243211, NM\_001562 and NM\_001386420). IL-18, structurally similar to IL-1 $\beta$ , is a member of IL-1 superfamily of cytokines. This cytokine, which is expressed by many human lymphoid and nonlymphoid cells, has an important role in inflammatory processes. IL-18 in combination with IL-12 can activate cytotoxic T cells (CTLs), as well as natural

killer (NK) cells, to produce IFN- $\gamma$  and, therefore, contributes to tumor immunity. Thus, without being bound by any particular theory of operation, it is believed that IL-18 can enhance the anti-tumor effects of the TIL compositions provided herein.

**[00282]** In some embodiments, the IL-18 is a tethered IL-18 (TeIL-18). In some embodiments, the TeIL-18 comprises the amino acid sequence of SEQ ID NO:132 (amino acids 1-18: human IgE signal sequence peptide; amino acids 176-200: peptide linker; amino acids 201-248: membrane anchor). In some embodiments, the TeIL-18 comprises the amino acid sequence of SEQ ID NO:134 (amino acids 1-18: human IgE signal sequence peptide; amino acids 175-199: peptide linker; amino acids 200-247: membrane anchor).

**[00283]** In some embodiments, the IL-18 is a full length IL-18, a fragment or a variant of IL-18. In some embodiments, the IL-18 is a human IL-18 or a variant human IL-18. In exemplary embodiments, the IL-18 is a biological active human IL-18 variant. In some embodiments, the IL-18 includes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 mutations as compared to a wild-type IL-18 (SEQ ID NO:75). In some embodiments, the bioactive variant is a decoy resistant IL-18 variant ("DR-IL18," or "DR-IL-18") that provides IL-18 signaling activity even in the presence of an inhibitory molecule such as IL-18 binding protein (IL-18BP). Exemplary IL-18 variants that can be included in the subject modified TILs described herein are shown below in Table 7. Additional IL-18 variants that can be included in the subject modified TILs are described in WO 2022/094473, which is incorporated by reference in its entirety and particular with respect to disclosures relating to variant DR-IL-18.

**[00284]** In some embodiments, the variant IL-18 includes a stability mutation pair selection from: C38S/C68S, C38S/C68G, C38S/C68A, C38S/C68D, and C38S/C68N [relative to the human wild-type IL-18 - SEQ ID NO: 75]. In some embodiments, the variant IL-18 includes mutations at amino acid positions M51 (e.g., M51E, M51R, M51K, M51T, M51D, or M51N), K53 (e.g., K53G, K53S, K53T, or K53R), Q56 (e.g., Q56G, Q56R, Q56L, Q56E, Q56A, Q56V, or Q56K), D110 (e.g., D110S, D110N, D110G, D110K, D110H, D110Q, or D110E) and N111 (e.g., N111G, N111R, N111S, N111D, N111H, or N111Y) in addition to a stabilizing mutation pair selected from: C38S/C68S, C38S/C68G, C38S/C68A, C38S/C68D, and C38S/C68N [relative to the human wild type IL-18 - SEQ ID NO: 75]. In some such cases the stabilized IL-18 variant polypeptide additionally includes a mutation at amino acid position S105 (e.g., S105D, S105A, S105N, S105R, S105D, or S105K); and in

some cases, further includes mutations at amino acid positions P57 (e.g., P57A, P57L, P57G, or P57K) and M60 (e.g., M60L, M60R, M60K, or M60Q).

TABLE 7 – IL-18 Related Sequences.

| Description         | Amino Acid Sequence                                                                                                                                                                                 |
|---------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-18         | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFQRSVPGHDNKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 75)     |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFARVPGHGRKTQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 76)      |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDSRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKSEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 77)     |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNR<br>PLFEDMTDSDSRDNAPRTIFIISKYSDSL<br>ARGLAVTISVKGEKISTLSCENKIISFKE<br>MNPPDNIKDTKSDIIFQRDVPGHSRK<br>MQFESSSYEGYFLACEKERDLFKLILK<br>KEDELGDRSIMFTVQNED (SEQ ID NO:<br>78) |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDSRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKA EKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 79)    |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDSRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKVEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 80)     |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDSRDNAPRTIFIISKYSDSLARGLAVT                                                                                                                                 |

| Description         | Amino Acid Sequence                                                                                                                                                                              |
|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                     | ISVKDEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 81)                                                                        |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDSRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKEEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 82) |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDSRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKEEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDEL (SEQ ID NO: 83)                 |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYKDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRDVPGHKHKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 84) |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYKDKQPRAKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRDVPGHKHKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTIQNED<br>(SEQ ID NO: 85) |
| Human IL-18 variant | RFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYKDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRDVPGHKHKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 86) |
| Human IL-18 variant | RFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRNVPGHKYKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 87) |

| Description         | Amino Acid Sequence                                                                                                                                                                                                                            |
|---------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-18 variant | YFGKLESQLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYKDKQPRTKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRRVPGHHNKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQKED<br>(SEQ ID NO: 88)                                               |
| Human IL-18 variant | YFGKLESRLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYKDKQPRAQAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRDVPGHKHKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTIQNED<br>(SEQ ID NO: 89)                                               |
| Human IL-18 variant | YFGKLESRLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISDYKDKQPRAXAA<br>VTISVKCEKISTLSCENKIISFKEMNPPDNIKD<br>TKSDIIFFQRDVPGHKHKMQFESSSYEGYFL<br>ACEKERDLFKLILKKEDELGDRSIMFTIQNED<br>(SEQ ID NO: 90)<br><br>X= any naturally occurring amino acid |
| Human IL-18 variant | YFGKHESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRDVPGHNNKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTTQNED<br>(SEQ ID NO: 91)                                               |
| Human IL-18 variant | YFGKIESKLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISKYKDKQPRAQAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFQRKVPGHQHMKQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQKED<br>(SEQ ID NO: 92)                                               |
| Human IL-18 variant | YFGKIESKLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYKDRQPRGKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFERDVPGHHHKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTIQNED<br>(SEQ ID NO: 93)                                               |
| Human IL-18 variant | YFGKIESKLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYKDKQPRGKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFQRDVPGHKHKMQFESSSYEGYFLACE                                                                                                   |



| Description         | Amino Acid Sequence                                                                                                                                                                               |
|---------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                     | KERDLFKLILKKEDELGDRSIMFTTQHED<br>(SEQ ID NO: 94)                                                                                                                                                  |
| Human IL-18 variant | YFGKIESKLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYKDKQPRAKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFQRRVPGHHHKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTIQKED<br>(SEQ ID NO: 95)  |
| Human IL-18 variant | YFGKIESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYKDKQPRGKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFQRDVPGHDYKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTIQKED<br>(SEQ ID NO: 96)  |
| Human IL-18 variant | YFGKYESRLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFQRDVPGHEHKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQKED<br>(SEQ ID NO: 97)   |
| Human IL-18 variant | HFGKYESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFQRDVPGHHNKMQLFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQKED<br>(SEQ ID NO: 98)  |
| Human IL-18 variant | RFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFQRDVPGHQHKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTAQKED<br>(SEQ ID NO: 99)   |
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISDYRDSQPRGRAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFKRNVPGHKYKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQHED<br>(SEQ ID NO: 100) |
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISNYRDSQPRGQAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS                                                                                         |

| Description         | Amino Acid Sequence                                                                                                                                                                                                                       |
|---------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                     | DIFFKRRVPGHNHMKMFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQKED<br>(SEQ ID NO: 101)                                                                                                                                                       |
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYKDSQPRGKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIFFQRDVPGHKHKMKMFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 102)                                         |
| Human IL-18 variant | RFGKHESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFERNVPGHKYKMKMFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 103)                                         |
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYRDSQPRKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIFFERDVPGHQHKMKMFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTIQXED<br>(SEQ ID NO: 104)<br>X= any naturally occurring amino acid |
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYRDSQPRTKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIFFQRNVPGHHDKMKMFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQHED<br>(SEQ ID NO: 105)                                         |
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYKDSQPRKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIFFERDVPGHQHKMKMFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTIQKED<br>(SEQ ID NO: 106)                                          |
| Human IL-18 variant | RFGKHESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYRDSQPRGKAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFERNVPGHKYKMKMFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 107)                                         |
| Human IL-18 variant | RFGKYESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISTYKDSQPRTKAVT<br>ISVKCEKISTLSCDNKIISFKEMNPPDNIKDTK<br>SDIIFQRDVPGHKHKMKMFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 108)                                         |

| Description         | Amino Acid Sequence                                                                                                                                                                                 |
|---------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-18 variant | RFGKLESRLSVIRNLNDQVLFIDQGNRPLFED<br>MTDSDCRDNAPRTIFIISTYRDSQPRTKAVTI<br>SVKCEKISTLSCENKIISFKEMNPPDNIKDTKS<br>DIIFFQRKVPGHNHKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQKED<br>(SEQ ID NO: 109)   |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISEYKDSELGRGAVT<br>ISVKCEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFPRAVPGHNRKVQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 110)   |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYKDSAGRGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFERDVPGHSNKVQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 111)   |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYGD SAARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRSVPGHKRKMVFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 112)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYGD SRGRGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFERDVPGHNSKRQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 113)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYGD SVPRGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARA VPGHSRKTQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 114) |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSGARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARA VPGHGRKTQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 115)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSKARGMAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARDVPGHSSKRQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 116)   |

| Description         | Amino Acid Sequence                                                                                                                                                                                |
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| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKCEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFQRDVPGHSRKMQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 117)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRNVPGHGRKMQFESSSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 118)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARSVPGHGRKTQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 119)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARDVPGHSGKRQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 120)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYTDSRPRGLAVT<br>ISVKCEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFERDVPGHSSKKQFESSSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 121)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYTDSRARGMAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFERDVPGHNDKRQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 122)  |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISRYKDSGKRGGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFRRSVPGHSRKVQFESSSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 123) |
| Human IL-18 variant | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYGD SGARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFERDVPGHSGKVQFESSSYEGYFLAC                                                       |

| Description                         | Amino Acid Sequence                                                                                                                                                                              |
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|                                     | EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 124)                                                                                                                                               |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYGDSRPRGMAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRAVPGHNRKMQFESSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 125) |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSLARGLAVT<br>ISVKCEKISTLSCENKIISFKEMNPPDNIKDTK<br>SDIIFFQRDVPGHNRKMQFESSYEGYFLACE<br>KERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 126) |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARVPGHGRKTQFESSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNE<br>(SEQ ID NO: 127)   |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFQRNVPGHGRKMQFESSYEGYFLA<br>CEKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 128) |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYGDSVPRGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARVPGHGRKTQFESSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 129)  |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARVPGHGRKTQFESSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 130)  |
| Human IL-18 variant                 | YFGKLESKLSVIRNLNDQVLFIDQGNRPLFE<br>DMTDSDCRDNAPRTIFIISKYSDSRARGLAV<br>TISVKCEKISTLSCENKIISFKEMNPPDNIKDT<br>KSDIIFFARDVPGHSGKRQFESSYEGYFLAC<br>EKERDLFKLILKKEDELGDRSIMFTVQNED<br>(SEQ ID NO: 131) |
| Tethered IL-18-Lr1-Ar2 (amino acid) | MDWTWILFLVAAATRVHSYFGKLESKLSVIRNL<br>NDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFI                                                                                                                           |

| Description                                             | Amino Acid Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|---------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                         | ISMYKDSQPRGMAVTISVKCEKISTLSCENKISF<br>KEMNPPDNIKDTKSDIIFQRSVPGHDNKMQF<br>ESSSYEGYFLACEKERDLFKLILKKEDELGDRSIM<br>FTVQNESSGGGGSGGGGSGGGGSGGGGSGG<br>GSLQLPSWAITLISVNGIFVICCLTYCFAPRCRE<br>RRRNERLRRESVRPV (SEQ ID NO:132)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| Tethered IL-18-Lr1-Ar2 (nucleotide)                     | ATGGATTGGACCTGGATTTTGTTCCTGGTGGC<br>TGCAGCCACTCGGGTCCACAGCTACTTCGGC<br>AAGCTGGAGAGTAACTTTCCGTGATCCGCA<br>ACCTCAACGACCAAGTGCTGTTTCATTGACCAG<br>GGCAACCGCCCCCTCTTTGAAGACATGACCG<br>ACAGCGATTGTCGTGACAATGCGCCCCGCAC<br>CATCTTTATAATTTCCATGTACAAGGATAGCC<br>AGCCGCGGGGAATGGCGGTAACGATCTCGG<br>TGAAGTGTGAAAAAATCTCGACCCTGTCATGC<br>GAGAACAAGATCATTTCTTCAAAGAGATGA<br>ATCCACCCGACAACATCAAGGACACCAAGTC<br>CGACATCATCTTCTCCAGCGCAGCGTGCCTG<br>GGCATGACAACAAGATGCAGTTCGAGAGCTC<br>GTCTTACGAGGGCTATTTCTGGCCTGCGAG<br>AAGGAGCGTGACCTGTTCAAGCTTATCCTGA<br>AGAAGGAGGACGAGCTGGGGGACCGCAGCA<br>TCATGTTTACAGTGCAGAACGAGGATTCTGG<br>TGGCGGCGGTAGCGGGGGCGGGGGTTCTGG<br>CGGCGGAGGCTCCGGTGGAGGAGGCTCCGG<br>CGGCGGTTGTTGCAGCTGCTGCCAGTTGG<br>GCCATCACTCTAATCTCCGTCAACGGCATCTT<br>CGTCATCTGTTGCCTGACCTACTGCTTTGCTCC<br>GAGGTGTAGAGAGCGACGCCGCAACGAGCG<br>CCTCCGCCGTGAGTCCGTGCGCCCTGTT (SEQ<br>ID NO:133) |
| Tethered DR-IL-18(6-27 variant)-Lr1-Ar2<br>(amino acid) | MDWTWILFLVAAATRVHSYFGKLESKLSVIRNL<br>NDQVLFIDQGNRPLFEDMTDSDCRDNAPRTIFI<br>ISKYSDSRARGLAVTISVKCEKISTLSCENKISFKE<br>MNPPDNIKDTKSDIIFARVPGHGRKTQFESSY<br>EGYFLACEKERDLFKLILKKEDELGDRSIMFTVQ<br>NEDSGGGGSGGGGSGGGGSGGGGSGGGSLQ<br>LLPSWAITLISVNGIFVICCLTYCFAPRCRERRRN<br>ERLRRESVRPV (SEQ ID NO:134)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
| Tethered DR-IL-18(6-27 variant)-Lr1-Ar2<br>(nucleotide) | ATGGATTGGACCTGGATCCTGTTCTGGTGG<br>CGGCGGCAACTCGCGTACATAGCTATTTCCG<br>CAAGCTGGAGAGCAAACTATCAGTGATCCGC<br>AACCTCAACGACCAGGTCTTGTATTGACCA<br>GGGCAACCGCCCCCTGTTGGAAGACATGACA<br>GACAGCGATTGTCGCGACAATGCTCCTCGCA                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |

| Description | Amino Acid Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|             | CCATCTTTATCATCTCCAAGTACTCCGATTCTC<br>GAGCCAGAGGTCTCGCCGTGACCATCTCCGT<br>GAAGTGCGAGAAGATATCCACTCTTTCATGC<br>GAGAACAAGATCATTTCTTCAAGGAGATGA<br>ATCCACCCGACAACATCAAGGACACCAAGTC<br>CGACATCATCTTCTTCGCTCGTGTGCCTGGCC<br>ACGGCCGCAAGACCCAGTTCGAGAGTTCAG<br>CTACGAGGGCTACTTCCTGGCCTGTGAGAAA<br>GAGCGGGACCTTTTCAAATTGATTCTGAAGA<br>AGGAGGACGAGCTGGGCGACCGTAGCATCA<br>TGTTTACGGTGCAGAACGAGGATTCTGGCGG<br>GGGCGGTTCTGGAGGGGGTGGGTGGGGCGG<br>TGGTGGCTCCGGGGGAGGCGGCTCCGGAGG<br>AGGCTCGCTGCAGCTGCTGCCAGTTGGGCC<br>ATCACGCTCATTTCTGTCAACGGCATCTTCGT<br>CATCTGTTGCCTGACCTACTGCTTTGCTCCGA<br>GGTGTAGGGAACGTCGTCGCAACGAGCGCCT<br>GCGCCGCGAGTCTGTTCGGCCGGTG (SEQ ID<br>NO:135) |

[00285] As used herein, “interleukin 21” “IL-21”, and “IL21” (e.g., Genbank Accession numbers: NM\_001207006 and NP\_001193935 (human); and NM\_0001291041 and NP\_001277970 (mouse)) all refer to a member of a cytokine that binds to IL-21 receptor and has potent regulatory effects on cells of the immune system, including natural killer (NK) cells and cytotoxic cells and binds to IL-21 receptor that can destroy virally infected or cancerous cells. Thus, without being bound by any particular theory of operation, it is believed that IL-21 can increase the survivability and/or anti-tumor effects of the TIL compositions provided herein.

[00286] In some embodiments, the IL-21 is a tethered IL-21. In some embodiments, the tethered IL-21 comprises the amino acid sequence of SEQ ID NO: 137 (amino acids 1-18: human IgE signal sequence peptide; amino acids 152-197: peptide linker; amino acids 198-245: membrane anchor).

[00287] In some embodiments, the IL-21 is a human IL-21 (SEQ ID NO: 136). In some embodiments, the IL-21 associated with the modified TIL is a full length IL-21, a fragment or a variant of IL-21. In some embodiments, the IL-21 is a human IL-21 or a variant human IL-21. In exemplary embodiments, the IL-21 is a biological active human IL-21 variant. In some embodiments, the IL-21 includes a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations as compared to a wild-type IL-21.

TABLE 8 – IL-21 Related Sequences.

| Description                 | Amino Acid Sequence                                                                                                                                                                                                                                                                               |
|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-21                 | QGQDRIIMIRMRLIDIVDQLKNYVNDLVPE<br>FLPAPEDVETNCEWSAFSCFQKAQLKSANT<br>GNNERIINVSIIKKLKRKPPSTNAGRQKHRL<br>TCPSCDSYEKKPPKEFLERFKSLQKMIHQH<br>LSSRTHGSEDS (SEQ ID NO:136)                                                                                                                              |
| Tethered IL-21 (amino acid) | MDWTWILFLVAAATRVHSQGQDRHMIRMRLQ<br>LIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS<br>AFSCFQKAQLKSANTGNNERIINVSIIKKLKRKPP<br>STNAGRQKHRLTCPSCDSYEKKPPKEFLERFKS<br>LLQKMIHQHLSRTHGSEDSSGGGGSGGGGSG<br>GGGGSGGGSGGGSGGGSGGGSGGGSGGGGS<br>GGGGSLQLPSWAITLISVNGIFVICCLTYCFAPR<br>CRERRRNERLRRESVRPV (SEQ ID NO:137) |

[00288] As used herein, “interleukin 2” “IL-2”, “IL2,” and “TCGF” (e.g., Genbank Accession numbers: NM\_000586 and NP\_000577 (human) all refer to a member of a cytokine that binds to IL-2 receptor. IL-2 enhances activation-induced cell death (AICD). IL-2 also promotes the differentiation of T cells into effector T cells and into memory T cells when the initial T cell is also stimulated by an antigen, thus helping the body fight off infections. Together with other polarizing cytokines, IL-2 stimulates naive CD4+ T cell differentiation into Th1 and Th2 lymphocytes and impedes differentiation into Th17 and follicular Th lymphocytes. IL-2 also increases the cell killing activity of both natural killer cells and cytotoxic T cells. Thus, without being bound by any particular theory of operation, it is believed that IL-2 can increase the survivability and/or anti-tumor effects of the TIL compositions provided herein.

[00289] In some embodiments, the IL-2 is a tethered IL-2. In some embodiments, the tethered IL-2 comprises the amino acid sequence of SEQ ID NO: 139 (amino acids 1-20: human IL-2 signal peptide; amino acids 154-178: peptide linker; amino acids 179-226: membrane anchor).

[00290] In some embodiments, the IL-2 is a human IL-2 (SEQ ID NO: 138). In some embodiments, the IL-2 associated with the modified TIL is a full length IL-2, a fragment or a variant of IL-2. In some embodiments, the IL-2 is a human IL-2 or a variant human IL-2. In exemplary embodiments, the IL-2 is a biological active human IL-2 variant. In some



embodiments, the IL-2 includes a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mutations as compared to a wild-type IL-2.

TABLE 9 – IL-2 Related Sequences.

| Description                        | Amino Acid Sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Human IL-2                         | MYRMQLLSICIALSLALVTNSAPTSSTKKTQ<br>LQLEHLLLDLQMLNGINNYKNPKLTRMLTF<br>KFYMPKKATELKHLQCLEEELKPLEEVLNL<br>AQSKNFHLRPRDLISNINVIVLELKGSETTFM<br>CEYADETATIVEFLNRWITFCQSIISTLT (SEQ<br>ID NO:138)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| Tethered IL-2-Lr1-Ar2 (amino acid) | MYRMQLLSICIALSLALVTNSAPTSSTKKTQLEHL<br>LLDLQMLNGINNYKNPKLTRMLTFKFYMPKKATEL<br>KHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINV<br>IVLELKGSETTFMCEYADETATIVEFLNRWITFCQSIIS<br>TLTSGGGGSGGGGSGGGGSGGGGSGGGSLQLLPS<br>WAITLISVNGIFVICLTFCFAPRCRERRRRNERLRRES<br>VRPV (SEQ ID NO:139)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
| Tethered IL-2-Lr1-Ar2 (nucleotide) | ATGTATCGGATGCAGCTGCTGTCTTGATTGCTCT<br>ATCACTGGCGCTGGTGACCAACTCCGCTCCTACTT<br>CCAGCTCGACCAAGAAGACCCAGCTCCAAGTTGA<br>GCACCTGCTGCTTGATCTTCAGATGATCCTGAACG<br>GCATCAACAACTACAAAAACCCCAAGCTGACCCG<br>CATGCTCACTTTCAAGTTCTACATGCCTAAAAAGG<br>CCACCGAGCTCAAGCACTTGCAGTGCCTGGAGGA<br>GGAGTTGAAGCCGTTGGAAGAGGTGCTCAACCTG<br>GCCCAGTCCAAGAATTTCCATCTAAGACCCGCGA<br>CCTGATCTCTAACATCAACGTGATTGTACTGGAGC<br>TGAAGGGCTCCGAGACCACCTTCATGTGCGAGTA<br>CGCGGACGAGACTGCAACCATCGTGGAGTTCCTG<br>AACCGCTGGATCACGTTCTGTGAGCATCATCTC<br>GACCCTTACATCTGGCGGCGGGGGTGGGGCGG<br>TGGGGGCTCTGGAGGAGGCGGTAGTGGGGGTG<br>GCGGCTCCGGTGGAGGCAGCCTGCAGCTGCTCCC<br>AAGTTGGGCCATCACCTGATTAGCGTCAATGGC<br>ATCTTCGTATATGTTGTCTGACCTACTGCTTGCC<br>CCCCGCTGCCGAGAACGTCGCCGCAACGAGCGGC<br>TGCGCAGGGAGTCCGTGCGCCCGGTGTGA (SEQ<br>ID NO:140) |

[00291] In some embodiments, the nucleic acid molecule provided herein further comprises a nucleotide sequence that encodes a truncated CD-19 (tCD19).

[00292] In some embodiments, the nucleic acid molecule provided herein further comprises a nucleotide sequence that encodes an shRNA. In some embodiments, the shRNA inhibits the expression of an immune checkpoint gene.

[00293] Non-limiting examples of immune checkpoint genes that may be silenced or inhibited by the shRNA include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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 the shRNA may be selected from the group comprising PD-1, CTLA-4, LAG-3, TIM-3, Cish, CBL-B, TIGIT, TET2, TGF $\beta$ , 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 the shRNA may be selected from the group comprising PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, TET2, CISH, TGF $\beta$ 2, PRA, CBLB, BAFF (BR3), and combinations thereof. Exemplary PD-1 shRNA sequences are provided in the table below.

TABLE 9 – PD-1 shRNA sequences

| Construct     | DNA sequence                                                            |
|---------------|-------------------------------------------------------------------------|
| PD-1 shRNA #1 | <u>GGATTTCCAGTGGCGAGAGAACTCGAGTTCTCTCGCCACTGGAAATCC</u> (SEQ ID NO:141) |
| PD-1 shRNA #2 | <u>CATTGTCTTTCCTAGCGGAATCTCGAGATTCCGCTAGGAAAGACAATG</u> (SEQ ID NO:142) |
| PD-1 shRNA #3 | <u>CAAGGCGCAGATCAAAGAGAGCTCGAGCTCTCTTTGATCTGCGCCTTG</u> (SEQ ID NO:143) |
| PD-1 shRNA #4 | <u>AAGGCGCAGATCAAAGAGAGCCTCGAGGCTCTCTTTGATCTGCGCCTT</u> (SEQ ID NO:144) |
| PD-1 shRNA #5 | <u>ATCGGAGAGCTTCGTGCTAAACTCGAGTTTAGCACGAAGCTCTCCGAT</u> (SEQ ID NO:145) |
| PD-1 shRNA #6 | <u>AGTATGCCACCATTGTCTTTCTCGAGGAAAGACAATGGTGGCATACT</u> (SEQ ID NO:146)  |
| PD-1 shRNA #7 | <u>GGATTTCCAGTGGCGAGAGAACTCGAGTTCTCTCGCCACTGGAAATCC</u> (SEQ ID NO:147) |

[00294] In some embodiments of the invention, the nucleic acid molecule disclosed herein further comprises long terminal repeat (LTR) sequences, for example, a 5' LTR sequence and

a 3' LTR sequence (Fig. 28). The LTR sequences are essential for viral genome integration into the host cell genome and viral gene expression in the host cell.

[00295] In an embodiment of the invention, the nucleic acid molecule disclosed herein is in the form of a recombinant lentiviral RNA molecule. For example, the recombinant lentiviral RNA molecule may comprise at least a portion of a lentivirus genome, including 5' and 3' LTRs. In some embodiments, the lentivirus genome may be modified to inhibit replication and limit pathogenicity, while retaining function. For example, the *env* gene, the *gag* gene, the *pol* gene, and the *rev* gene may be removed from the lentivirus genome and included in helper plasmids. In some embodiments, the recombinant lentiviral RNA molecule is comprised by a lentiviral vector or a recombinant lentiviral particle as described elsewhere in this disclosure.

[00296] In another embodiment of the invention, the nucleic acid molecule disclosed herein is in the form of a recombinant lentiviral proviral DNA molecule. For example, the recombinant lentiviral proviral DNA molecule is comprised by a host cell, e.g., a gene-edited TIL as disclosed herein. In some embodiments, the recombinant lentiviral proviral DNA molecule is integrated into the genome of the host cell, e.g., a gene-edited TIL as disclosed herein. In some embodiments, recombinant lentiviral proviral DNA molecule is comprised by a packaging cell line as described elsewhere in this disclosure. In some embodiments, the recombinant lentiviral proviral DNA molecule is integrated into the genome of the packaging cell line.

TABLE 10 – Exemplary nucleic acid molecule sequences

| C<br>on<br>str<br>uc<br>t                                      | DNA sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |
|----------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| N<br>F<br>A<br>T-<br>Te<br>IL<br>-<br>12<br>-<br>E<br>F-<br>la | AGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAGGAAAACT<br>GTTTCATACAGAAGGCGTCAATTAGGAGGAAAACTGTTTCATACAGAAG<br>GCGTCAATTGGTCCCATCGAATTAGGAGGAAAACTGTTTCATACAGAAG<br>GCGTCAATTAGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAG<br>GAAAACTGTTTCATACAGAAGGCGTCAATTGGTCCCGGGACATTTTGAC<br>ACCCCCATAATATTTTCCAGAATTAACAGTATAAATTGCATCTCTTGTTT<br>AAGAGTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCC<br>TGGACCGGTTCTAGAGCGCTTTAATTAAGCCACCATGGATTGGACCTGGAT<br>TCTGTTCTCGTGGCCGCCGCTACCCGCGTGCACTCCATCTGGGAGCTGAA<br>GAAAGACGTGTACGTGGTGGGAATTGGATTGGTACCCCGACGCCCCAGGCG<br>AGATGGTGGTTCTGACTTGCGACACGCCGAGGAGGACGGCATCACCTGG<br>ACGCTGGACCAAGAGCAGCGAAGTGCTGGGCTCTGGTAAGACCTTAACCAT |

|    |                                                      |
|----|------------------------------------------------------|
| -  | CCAGGTCAAGGAGTTCGGTGATGCAGGACAGTACACGTGTCACAAGGGCG   |
| tC | GGGAGGTCTTGTCCCATAGCCTGCTGCTCCTGCACAAGAAGGAAGACGGC   |
| D  | ATCTGGTCTACGGATATCCTTAAAGACCAGAAGGAGCCCAAGAACAAAAC   |
| 19 | CTTCCTGCGATGCGAGGCTAAGAATTACTCCGGGCGCTTTACCTGTTGGTG  |
|    | GCTCACAACCATCTCGACCGACCTGACCTTCTCCGTGAAGTCTTCCAGGGG  |
|    | ATCTTCTGATCCGCAAGGCGTTACCTGCGGGGCGCTACCTGTGCGGTGA    |
|    | GCGCGTCCGTGGTGACAACAAGGAGTACGAGTACTCGGTGGAATGCCAGG   |
|    | AGGACAGCGCCTGCCCAGCGGCCGAAGAGTCCTTGCCCATCGAGGTGATG   |
|    | GTGGACGCTGTACACAAGCTCAAGTACGAGAACTACACCTCCTCCTTCTT   |
|    | ATACGCGACATCATCAAGCCTGACCCCCCTAAAAACCTGCAGCTGAAGCC   |
|    | CCTTAAAAACTCTCGACAGGTGAGGTATCCTGGGAGTACCCCGACACCT    |
|    | GGTCTACTCCTCATTCGTATTTAGCCTGACCTTCTGCGTGCAGGTGCAGG   |
|    | GCAAGTCCAAGCGCGAGAAGAAGGACCGCGTGTTACCGACAAAACAG      |
|    | CGCGACTGTCAATTTGTCGGAAGAACGCGAGCATCTCCGTACGGGCACAGG  |
|    | ATCGCTATTACTCCAGCAGTTGGTCAGAGTGGGCTTCGGTTCGGTGTCTG   |
|    | GAGGAGGTGGCGGGCGCTACGCAACCTGCCCGTGGCGACGCCGAGCCCG    |
|    | GGTATGTTTCTTGTCTGCACCACTCTCAGAACTTGCTCCGTGCCGTCTCG   |
|    | AATATGCTGCAGAAGGCCCCGACACTCTGGAGTTCTATCCATGCACTAG    |
|    | TGAGGAGATCGATCATGAGGACATCACCAAGGACAAGACATCCACCGTGG   |
|    | AGGCTTGTCTGCCGCTGGAGTTGACAAAGAACGAGAGCTGCCTCAATAGC   |
|    | CGCGAGACTTCCTTCATCACCAACGGCTCCTGCCTCGCGAGCCGTAAGACC  |
|    | TCCTTTATGATGGCGCTGTGCCTGTCAAGTATCTACGAAGATCTGAAAATG  |
|    | TATCAGGTGGAGTTCAAACTATGAACGCAAAACTCCTGATGGATCCCAA    |
|    | GCGCCAAATTTTCTTGGACCAGAACATGCTTGCCGTGATCGACGAGCTGAT  |
|    | GCAGGCCCTGAACCTCAACTCCGAGACCGTCCCTCAGAAAGTCGTCTCTGG  |
|    | AGGAACCCGACTTTTACAAGACAAAGATCAAGCTGTGCATCCTGCTACAC   |
|    | GCGTTCCGCATTCGGGCCGTGACCATTGACCGCGTGATGTCCTACCTGAAT  |
|    | GCCTGCTCCGGCGGGGGGGGCTCCGGTGGGGGGCGGCTCTGGCGGAGGCGG  |
|    | CTCAGGTGGCGGCGGCTCCGGCGGAGGTTGCTTCAGCTTCTGCCAGGTG    |
|    | GGCAATCACTCTGATTTCCGTCAACGGAATCTTCGTATCTGCTGCCTGAC   |
|    | CTACTGCTTTGCTCCCAGATGTAGAGAGCGCCGCCGCAACGAGCGCCTGC   |
|    | GTCGTGAGAGCGTGAGGCCTGTGTGAGAATTCCGCCCCCCCCCCCCCG     |
|    | GGCAGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTG    |
|    | GGCAATTGATCCGGTGCCTAGAGAAGGTGGCGCGGGGTAACTGGGAAA     |
|    | GTGATGTGCTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGT  |
|    | ATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCG  |
|    | CCAGAACACAGGCTTGATCACGCGTGCCACCATGCCCCCGCCTAGACTAC   |
|    | TCTTTTCTCTGCTTTTCTGACTCCGATGGAGGTGCGCCCCGAGGAGCCAT   |
|    | TAGTGGTGAAGGTGGAGGAAGGTGACAACGCCGTGTTACAGTGCCTGAAG   |
|    | GGAACCTCCGACGGCCCTACTCAGCAGCTGACCTGGTCTCGCGAGAGCCC   |
|    | CCTGAAGCCGTTCTTGAACTCAGTCTTGGCCTGCCCGGCCCTAGGCATCCA  |
|    | CATGAGGCCTCTGGCGATTGGCTCTTTATTTTCAACGTGTCCCAGCAGAT   |
|    | GGGCGGCTTCTATCTATGCCAGCCTGGCCACCCCTCTGAAAAGGCTTGGCA  |
|    | ACCAGGTTGGACTGTCAACGTGGAGGGCAGCGGGGAGCTGTTACAGGTGGA  |
|    | ACGTGTCCGACCTGGGCGGTCTCGGGTGCGGGCTTAAAAACCGCAGCTCG   |
|    | GAGGGGCCAGCTCCCCATCCGGTAAGCTCATGTCCCCTAAGCTGTACGTC   |
|    | TGGGCCAAGGACCGCCCTGAGATCTGGGAAGGAGAGCCTCCGTGCGTGCC   |
|    | CCGCGTGATTCGCTCAACCAGAGCCTGTCCCAGGACCTACGATGGCAC     |
|    | CCGGTAGCACCCCTGTGGTTATCCTGCGGCGTCCCCCCCCGACTCCGTGTCC |
|    | GTGGACCCCTGAGTTGGACACATGTCCACCCCAAGGGCCCTAAGTCATTG   |

|                                                                                                        |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|--------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                                                                        | TTGTCTTTGGAGCTGAAAGATGACCGGCCGGCTCGTGACATGTGGGTGAT<br>GGAGACCGGCCTGCTGCTGCCGCGCGCCACGGCCCAGGATGCGGGGAAGT<br>ACTACTGTCACCGCGGCAACCTGACCATGTTCGTTCCACTTGGAGATCACCG<br>CGCGCCAGTGCTGTGGCATTGGCTTCTCCGAACCGGCGGATGGAAGGTT<br>TCCGCTGTACCCCTGGCCTACCTGATCTTTTGTCTGTGCTCCCTGGTTGGTA<br>TCCTGCACCTGCAGCGCGCTCTGGTACTGCGCCGGAAGCGCtaa, (SEQ ID<br>NO:148)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| N<br>F<br>A<br>T-<br>Te<br>IL<br>12<br>-<br>E<br>F-<br>la<br>-<br>tC<br>D<br>19<br>-<br>Te<br>IL<br>-2 | AGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAGGAAAACT<br>GTTTCATACAGAAGGCGTCAATTAGGAGGAAAACTGTTTCATACAGAAG<br>GCGTCAATTGGTCCCATCGAATTAGGAGGAAAACTGTTTCATACAGAAG<br>GCGTCAATTAGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAG<br>GAAAACTGTTTCATACAGAAGGCGTCAATTGGTCCCGGGACATTTTGAC<br>ACCCCCATAATATTTTCCAGAATTAACAGTATAAATTGCATCTCTTGTTT<br>AAGAGTTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCC<br>TGGACCGGTTCTAGAGCGCTTTAATTAAGCCACCATGGATTGGACCTGGAT<br>TCTGTTCCCTCGTGGCCGCCGCTACCCGCGTGCACTCCATCTGGGAGCTGAA<br>GAAAGACGTGTACGTGGTGGAAATTGGATTGGTACCCCGACGCCCCAGGCG<br>AGATGGTGGTTCTGACTTGCACACGCCGGAGGAGGACGGCATCACCTGG<br>ACGCTGGACCAGAGCAGCGAAGTGCTGGGCTCTGGTAAGACCTTAACCAT<br>CCAGGTCAAGGAGTTCGGTGATGCAGGACAGTACACGTGTCACAAGGGCG<br>GGGAGGTCTTGTCCCATAGCCTGCTGCTCCTGCACAAGAAGGAAGACGGC<br>ATCTGGTCTACGGATATCCTTAAAGACCAGAAGGAGCCCAAGAACAAAAC<br>CTTCCTGCGATGCGAGGCTAAGAATTACTCCGGGCGCTTTACCTGTTGGTG<br>GCTCACAACCATCTCGACCGACCTGACCTTCTCCGTGAAGTCTTCCAGGGG<br>ATCTTCTGATCCGCAAGGCGTTACCTGCGGGGGCCGCTACCCTGTCCGGTGA<br>GCGCGTCCGTGGTGACAACAAGGAGTACGAGTACTCGGTGGAATGCCAGG<br>AGGACAGCGCCTGCCCAGCGGCCGAAGAGTCTTGGCCATCGAGGTGATG<br>TGGGACGCTGTACACAAGCTCAAGTACGAGAACTACACCTCCTCCTTTT<br>ATACGCGACATCATCAAGCCTGACCCCCCTAAAACTGCAGCTGAAGCC<br>CCTTAAAACTCTCGACAGGTGAGGTATCCTGGGAGTACCCCGACACCT<br>GGTCTACTCCTCATTTCGATTTTACGCTGACCTTCTGCGTGACGGTGCAGG<br>GCAAGTCCAAGCGCGAGAAGAAGGACCGCGTGTTACCGACAAAACAG<br>CGCGACTGTCATTTGTGCGAAGAACGCGAGCATCTCCGTACGGGCACAGG<br>ATCGCTATTACTCCAGCAGTTGGTCAGAGTGGGCTTCGGTTCCTGTCTG<br>GAGGAGGTGGCGGCGGCTCACGCAACCTGCCCGTGGCGACGCCGGACCCG<br>GGTATGTTTCCTTGTCTGCACCACTCTCAGAACTTGCTCCGTGCCGTCTCG<br>AATATGCTGCAGAAGGCCCCGCCAGACTCTGGAGTTCTATCCATGCACTAG<br>TGAGGAGATCGATCATGAGGACATCACCAAGGACAAGACATCCACCGTGG<br>AGGCTTGTCTGCCGCTGGAGTTGACAAAGAACGAGAGCTGCCTCAATAGC<br>CGCGAGACTTCCTTCATCACCAACGGCTCCTGCCTCGCGAGCCGTAAGACC<br>TCCTTTATGATGGCGCTGTGCCTGTCAAGTATCTACGAAGATCTGAAAATG<br>TATCAGGTGGAGTTCAAACTATGAACGCAAACTCCTGATGGATCCCAA<br>GCGCCAAATTTTCTTGGACCAGAACATGCTTGCCGTGATCGACGAGCTGAT<br>GCAGGCCCTGAACTTCAACTCCGAGACCGTCCCTCAGAAGTCTGTCTCTGG<br>AGGAACCCGACTTTTACAAGACAAAGATCAAGCTGTGCATCCTGCTACAC<br>GCGTTCCGCATTCGGGCCGTGACCATTGACCGCGTGATGTCCTACCTGAAT<br>GCCTCGTCCGCGGGGGGGGCTCCGGTGGGGGCGGCTCTGGCGGAGGCGG<br>CTCAGGTGGCGGCGGCTCCGGCGGAGGTTTCGCTTCAGCTTCTGCCAGTTG<br>GGCAATCACTCTGATTTCCGTCAACGGAATCTTCGTATCTGCTGCCTGAC<br>CTACTGCTTTGCTCCCAGATGTAGAGAGCGCCGCCGCAACGAGCGCCTGC |

|                                          |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
|------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                          | <p>GTCGTGAGAGCGTGAGGCCTGTGTGAGAATTCCGCCCCCCCCCCCCCGG<br/> GGCAGAGCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTC<br/> GGCAATTGATCCGGTGCCTAGAGAAGGTGGCGCGGGGTAACTGGGAAA<br/> GTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGGTGGGGGAGAACCGT<br/> ATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTTCGCAACGGGTTTGCCG<br/> CCAGAACACAGGCTTGATCACGCGTGCCACCATGCCCCGCCTAGACTAC<br/> TCTTTTTCTGCTTTTTCTGACTCCGATGGAGGTGCGCCCCGAGGAGCCAT<br/> TAGTGGTGAAGGTGGAGGAAGGTGACAACGCCGTGTTACAGTGCCTGAAG<br/> GGAACCTCCGACGGCCCTACTCAGCAGCTGACCTGGTCTCGCGAGAGCCC<br/> CCTGAAGCCGTTCTTGAAACTCAGTCTTGGCCTGCCCGGCCCTAGGCATCCA<br/> CATGAGGCCTCTGGCGATTGCGCTCTTTATTTTCAACGTGTCCCAGCAGAT<br/> GGGCGGCTTCTATCTATGCCAGCCTGGCCCACCCTCTGAAAAGGCTTGCCA<br/> ACCAGGTTGGACTGTCAACGTGGAGGGCAGCGGGGAGCTGTTACAGGTGGA<br/> ACGTGTCCGACCTGGGCGGTCTCGGGTGCGGGCTTAAAAACCGCAGCTCG<br/> GAGGGCCCCAGCTCCCCATCCGGTAAGCTCATGTCCCCTAAGCTGTCGTC<br/> TGGGCCAAGGACCGCCCTGAGATCTGGGAAGGAGAGCCTCCGTGCGTGCC<br/> CCGCGTGATTTCGCTCAACCAGAGCCTGTCCCAGGACCTCACGATGGCAC<br/> CCGGTAGCACCCCTGTGGTTATCCTGCGGCGTCCCCCCCCGACTCCGTGTCCC<br/> GTGGACCCCTGAGTTGGACACATGTCCACCCCAAGGGCCCTAAGTCATTG<br/> TTGTCTTTGGAGCTGAAAGATGACCGGCCGGCTCGTGACATGTGGGTGAT<br/> GGAGACCGGCCTGCTGCTGCCGCGCGCCACGGCCCAGGATGCGGGGAAGT<br/> ACTACTGTCACCGCGGCAACCTGACCATGTGCTTCCACTTGAGATCACCG<br/> CGCGCCAGTGCTGTGGCATTGGCTTCTCCGAACCGGCGGATGGAAGGTT<br/> TCCGCTGTACCCCTGGCCTACCTGATCTTTTGTCTGTGCTCCCTGGTTGGTA<br/> TCCTGCACCTGCAGCGCGCTCTGGTACTGCGCCGGAAGCGCCGCGCCAAG<br/> CGCAGCGGCTCGGGCGCCACTAACTTTTCACTCTTGAAGCAGGCCGGTGA<br/> TGTGGAGGAGAACCCGGGCCCTATGTATCGTATGCAGCTTCTGTCTGTAT<br/> TGCATTTTCTCTGGCGCTGGTGACCAACTCCGCGCCAACCTCCAGCTCGAC<br/> CAAGAAGACCCAGCTACAACCTGGAGCACCTTTACTGGACCTGCAGATGA<br/> TCCTGAATGGCATTAAACAACCTACAAGAATCCAAAGCTGACTCGCATGCTC<br/> ACGTTCAAGTTCTACATGCCCAAGAAGGCCACCGAGCTTAAGCACTTGCA<br/> GTGCCTGGAGGAGGAGCTGAAGCCCCCTGGAAGAGGTGCTAAACCTGGCTC<br/> AGAGCAAAAACCTTCCATCTCCGGCCCAGAGACCTCATCTCTAATATCAAC<br/> GTGATTGTTCTGGAACCTGAAAGGCTCCGAAACCACCTTCATGTGCGAGTA<br/> CGCGGACGAGACCGTACCATCGTGGAGTTCCTGAACCGCTGGATCACAT<br/> TCTGTACGTCTATCATCTCTACTTTGACCAGCGGGGGTGGGGGCTCCGGCG<br/> GTGGGGGTTCTGGCGGCGGCGGATCCGGAGGAGGGGGCTCCGGTGGAGG<br/> CTCCCTCCAGCTGCTGCCAGTTGGGCCATCACCTGATATCCGTCAACGG<br/> CATCTTCGTATCTGTTGCCCTGACCTACTGCTTTGCTCCTAGGTGTAGGGA<br/> GCGCCGCGCAACGAGCGCCTGCGCCGCGAGAGCGTGCGCCCGGTAtaa<br/> (SEQ ID NO:149)</p> |
| N<br>F<br>A<br>T-<br>Te<br>IL<br>12<br>- | <p>AGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAGGAAAACT<br/> GTTTCATACAGAAGGCGTCAATTAGGAGGAAAACTGTTTCATACAGAAG<br/> GCGTCAATTGGTCCCATCGAATTAGGAGGAAAACTGTTTCATACAGAAG<br/> GCGTCAATTAGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAG<br/> GAAAAACTGTTTCATACAGAAGGCGTCAATTGGTCCCGGGACATTTTGAC<br/> ACCCCCATAATATTTTCCAGAAATTAACAGTATAAATTGCATCTCTTGTT<br/> AAGAGTTCCCTATCACTCTCTTAATCACTACTCACAGTAACCTCAACTCC<br/> TGGACCGGTTCTAGAGCGCTTTAATTAAGCCACCATGGATTGGACCTGGAT</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |

|    |                                                       |
|----|-------------------------------------------------------|
| E  | TCTGTTCCCTCGTGGCCGCCGCTACCCGCGTGCACCTCCATCTGGGAGCTGAA |
| F1 | GAAAGACGTGTACGTGGTGGGAATTGGATTGGTACCCCGACGCCCCAGGCG   |
| a- | AGATGGTGGTTCTGACTTGCGACACGCCGGAGGAGGACGGCATCACCTGG    |
| tC | ACGCTGGACCAGAGCAGCGAAGTGCTGGGCTCTGGTAAGACCTTAACCAT    |
| D  | CCAGGTCAAGGAGTTCGGTGATGCAGGACAGTACACGTGTCACAAGGGCG    |
| 19 | GGGAGGTCTTGTCCCATAGCCTGCTGCTCCTGCACAAGAAGGAAGACGGC    |
| -  | ATCTGGTCTACGGATATCCTTAAAGACCAGAAGGAGCCCAAGAACAAAAC    |
| Te | CTTCCTGCGATGCGAGGCTAAGAATTACTCCGGGCGCTTTACCTGTTGGTG   |
| IL | GCTCACAAACCATCTCGACCGACCTGACCTTCTCCGTGAAGTCTTCCAGGGG  |
| -  | ATCTTCTGATCCGCAAGGCGTTACCTGCGGGGCCGCTACCTGTGCGGTGA    |
| 15 | GCGCGTCCGTGGTGACAACAAGGAGTACGAGTACTCGGTGGAATGCCAGG    |
|    | AGGACAGCGCCTGCCCAGCGGCCGAAGAGTCCCTTGCCCATCGAGGTGATG   |
|    | GTGGACGCTGTACACAAGCTCAAGTACGAGAACTACACCTCCTCCTTCTT    |
|    | ATACGCGACATCATCAAGCCTGACCCCCCTAAAAACCTGCAGCTGAAGCC    |
|    | CCTTAAAAACTCTCGACAGGTCGAGGTATCCTGGGAGTACCCCGACACCT    |
|    | GGTCTACTCCTCATTCGTATTTACGCTGACCTTCTGCGTGACAGGTGCAGG   |
|    | GCAAGTCCAAGCGCGAGAAGAAGGACCGCGTGTTACCGACAAAACAG       |
|    | CGCGACTGTCATTTGTCGGAAGAACGCGAGCATCTCCGTACGGGCACAGG    |
|    | ATCGCTATTACTCCAGCAGTTGGTCAGAGTGGGCTTCGGTTCCTGTTCTG    |
|    | GAGGAGGTGGCGGGCGGCTCACGCAACCTGCCCGTGGCGACGCCGGACCCG   |
|    | GGTATGTTTCCTTGTCTGCACCACTCTCAGAACTTGCTCCGTGCCGTCTCG   |
|    | AATATGCTGCAGAAGGCCCGCCAGACTCTGGAGTTCTATCCATGCACTAG    |
|    | TGAGGAGATCGATCATGAGGACATCACCAAGGACAAGACATCCACCGTGG    |
|    | AGGCTTGTCTGCCGCTGGAGTTGACAAAAGAACGAGAGCTGCCTCAATAGC   |
|    | CGCGAGACTTCCTTCATCACCAACGGCTCCTGCCTCGCGAGCCGTAAGACC   |
|    | TCCTTTATGATGGCGCTGTGCCTGTCAAGTATCTACGAAGATCTGAAAATG   |
|    | TATCAGGTGGAGTTCAAAACCTATGAACGCAAAACTCCTGATGGATCCCAA   |
|    | GCGCCAAATTTTCTTGGACCAGAACATGCTTGCCGTGATCGACGAGCTGAT   |
|    | GCAGGCCCTGAACCTTCAACTCCGAGACCGTCCCTCAGAAGTCGTCTCTG    |
|    | AGGAACCCGACTTTTACAAGACAAAGATCAAGCTGTGCATCCTGCTACAC    |
|    | GCGTTCCGCATTCGGGCCGTGACCATTGACCGCGTGATGTCCTACCTGAAT   |
|    | GCCTCGTCCGGCGGGGGGGGCTCCGGTGGGGGCGGCTCTGGCGGAGGCGG    |
|    | CTCAGGTGGCGGCGGCTCCGGCGGAGGTTCTGCTTCAGCTTCTGCCAGTTG   |
|    | GGCAATCACTCTGATTTCCGTCAACGGAATCTTCGTATCTGCTGCCTGAC    |
|    | CTACTGCTTTGCTCCCAGATGTAGAGAGCGCCGCCGCAACGAGCGCCTGC    |
|    | GTCGTGAGAGCGTGAGGCCTGTGTGAGAATTCCGCCCCCCCCCCCCCG      |
|    | GGCAGAGCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTC      |
|    | GGCAATTGATCCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAA     |
|    | GTGATGTGCTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGT   |
|    | ATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCG   |
|    | CCAGAACACAGGCTTGATCACGCGTGCCACCATGCCCCCGCCTAGACTAC    |
|    | TCTTTTCTGCTTTTCTGACTCCGATGGAGGTGCGCCCCGAGGAGCCAT      |
|    | TAGTGGTGAAGGTGGAGGAAGGTGACAACGCCGTGTTACAGTGCCTGAAG    |
|    | GGAACCTCCGACGGCCCTACTCAGCAGCTGACCTGGTCTCGCGAGAGCCC    |
|    | CCTGAAGCCGTTCTTGAAACTCAGTCTTGGCTGCCCGGCTAGGCATCCA     |
|    | CATGAGGCCTCTGGCGATTTGGCTCTTTATTTTCAACGTGTCCAGCAGAT    |
|    | GGGCGGCTTCTATCTATGCCAGCCTGGCCACCCTCTGAAAAGGCTTGGCA    |
|    | ACCAGGTTGGACTGTCAACGTGGAGGGCAGCGGGGAGCTGTTACGGTGA     |
|    | ACGTGTCCGACCTGGGCGGTCTCGGGTGCGGGCTTAAAAACCGCAGCTCG    |
|    | GAGGGGCCAGCTCCCCATCCGGTAAGCTCATGTCCCCTAAGCTGTACGTC    |

|                                                                                                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|----------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                                                                                                      | TGGGCCAAGGACCGCCCTGAGATCTGGGAAGGAGAGCCTCCGTGCGTGCC<br>CCCGCGTGATTCGCTCAACCAGAGCCTGTCCCAGGACCTCACGATGGCAC<br>CCGGTAGCACCCCTGTGGTTATCCTGCGGGCTCCCCCGACTCCGTGTCCC<br>GTGGACCCCTGAGTTGGACACATGTCCACCCCAAGGGCCCTAAGTCATTG<br>TTGTCTTTGGAGCTGAAAGATGACCGGCCGGCTCGTGACATGTGGGTGAT<br>GGAGACCGGCCTGCTGCTGCCGCGGCCACGGCCCAGGATGCGGGGAAGT<br>ACTACTGTCACCGCGGCAACCTGACCATGTCGTTCCACTTGGAGATCACCG<br>CGCGCCAGTGCTGTGGCATTGGCTTCTCCGAACCGGCGGATGGAAGGTT<br>TCCGCTGTCACCTGGCCTACCTGATCTTTTGTCTGTGCTCCCTGGTTGGTA<br>TCCTGCACCTGCAGCGCGCTCTGGTACTGCGCCGGAAGCGCCGTGCTAAG<br>CGCAGCGGCTCCGGAGGCCACCAACTTTTCACTCCTTAAGCAGGCTGGAGA<br>CGTGGAGGAGAATCCTGGCCCAATGGATTGGACCTGGATCCTGTTCTTG<br>TGGCAGCGGCCACTCGCGTTCACCTCGAATTGGGTCAACGTGATCTCGGAC<br>CTTAAGAAGATCGAGGACTTAATCCAATCCATGCACATTGACGCGACACT<br>GTACCCGAGAGCGATGTGCACCCCTCTTGTAAGGTGACCGCCATGAAAT<br>GTTTCTTGCTCGAGCTGCAGGTCACTCTCTGGAATCTGGCGACGCGTCCA<br>TCCATGACACCGTGGAGAACCTGATCATCCTGGCCAACAACCTCTCTATCCT<br>CCAATGGCAACGTGACTGAGAGCGGATGCAAGGAGTGTGAAGAGCTGGA<br>GGAGAAGAACATAAAAGAGTTCTTGCAGAGCTTCGTCCACATCGTGCAGA<br>TGTTTATTAACACGAGCTCCGGCGGTGGCGGCAGCGCGGGGGCGGCTCC<br>GGTGGCGGGGGTAGCGGAGGGGGTGGCTCTGGTGGCGGGTCTTGCAGCT<br>GCTGCCCAGTTGGGCCATCACCCCTCATCTCGGTGAACGGCATCTTCGTCAT<br>TTGTTGCCTGACGTACTGCTTCGCTCCGAGATGCCGAGAACGCCGTGCAAC<br>CGAGCGCCTGCGCAGGGAGAGTGTACGGCCTGTGtaa (SEQ ID NO:150)                                                 |
| N<br>F<br>A<br>T-<br>Te<br>IL<br>12<br>-<br>E<br>Fl<br>a-<br>tC<br>D<br>19<br>-<br>P<br>D-<br>ls<br>h<br>R<br>N<br>A | AGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAGGAAAACT<br>GTTTCATACAGAAGGCGTCAATTAGGAGGAAAACTGTTTCATACAGAAG<br>GCGTCAATTGGTCCCATCGAATTAGGAGGAAAACTGTTTCATACAGAAG<br>GCGTCAATTAGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAG<br>GAAAACTGTTTCATACAGAAGGCGTCAATTGGTCCCGGGACATTTTGAC<br>ACCCCCATAATATTTTCCAGAATTAACAGTATAAATTGCATCTCTTGTTT<br>AAGAGTTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCC<br>TGGACCGGTTCTAGAGCGCTTTAATTAAGCCACCATGGATTGGACCTGGAT<br>TCTGTTCCCTCGTGGCCGCCGCTACCCGCGTGCACCTCCATCTGGGAGCTGAA<br>GAAAGACGTGTACGTGGTGGAAATTGGATTGGTACCCCGACGCCCCAGGCG<br>AGATGGTGGTTCTGACTTGCGACACGCCGGAGGAGGACGGCATCACCTGG<br>ACGCTGGACCAGAGCAGCGAAGTGCTGGGCTCTGGTAAGACCTTAACCAT<br>CCAGGTCAAGGAGTTCGGTGATGCAGGACAGTACACGTGTCACAAGGGCG<br>GGGAGGTCTTGTTCCATAGCCTGCTGCTCCTGCACAAGAAGGAAGACGGC<br>ATCTGGTCTACGGATATCCTTAAAGACCAGAAGGAGCCCAAGAACAAAAC<br>CTTCCTGCGATGCGAGGCTAAGAATTACTCCGGGCGCTTTACCTGTTGGTG<br>GCTCACAACCATCTCGACCGACCTGACCTTCTCCGTGAAGTCTTCCAGGGG<br>ATCTTCTGATCCGCAAGGCGTTACCTGCGGGGCGCTACCTGTGCGGTGA<br>GCGCGTCCGTGGTGACAACAAGGAGTACGAGTACTCGGTGGAATGCCAGG<br>AGGACAGCGCCTGCCAGCGGCCGAAGAGTCCTTGCCCATCGAGGTGATG<br>GTGGACGCTGTACACAAGCTCAAGTACGAGAACTACACCTCCTCCTTCTTT<br>ATACGCGACATCATCAAGCCTGACCCCCCTAAAAACCTGCAGCTGAAGCC<br>CCTTAAAAACTCTCGACAGGTGAGGTATCCTGGGAGTACCCCGACACCT<br>GGTCTACTCCTCATTTCGTAATTCAGCCTGACCTTCTGCGTGCAGGTGCAGG<br>GCAAGTCCAAGCGCGAGAAGAAGGACCGCGTGTTCACCGACAAAACAG |



CGCGACTGTCATTTGTCGGAAGAACGCGAGCATCTCCGTACGGGCACAGG  
ATCGCTATTACTCCAGCAGTTGGTCAGAGTGGGCTTCGGTTCCGTGTTCTG  
GAGGAGGTGGCGGCGGCTCACGCAACCTGCCCGTGGCGACGCCGACCCG  
GGTATGTTTCCTTGTCTGCACCACTCTCAGAACTTGCTCCGTGCCGTCTCG  
AATATGCTGCAGAAGGCCCCGCCAGACTCTGGAGTTCTATCCATGCACTAG  
TGAGGAGATCGATCATGAGGACATCACCAAGGACAAGACATCCACCGTGG  
AGGCTTGTCTGCCGCTGGAGTTGACAAAGAACGAGAGCTGCCTCAATAGC  
CGCGAGACTTCCTTCATCACCAACGGCTCCTGCCTCGCGAGCCGTAAGACC  
TCCTTTATGATGGCGCTGTGCCTGTCAAGTATCTACGAAGATCTGAAAATG  
TATCAGGTGGAGTTCAAAACTATGAACGCAAAACTCCTGATGGATCCCAA  
GCGCCAAATTTTCTTGGACCAGAACATGCTTGCCGTGATCGACGAGCTGAT  
GCAGGCCCTGAAC TTCAACTCCGAGACCGTCCCTCAGAAAGTCGTCTCTGG  
AGGAACCCGACTTTTACAAGACAAAGATCAAGCTGTGCATCCTGCTACAC  
GCGTTCCGCATTTCGGGCCGTGACCAATTGACCGCGTGATGTCCTACCTGAAT  
CCTGATCCCGCGGGGGGGGCTCCGGTGGGGGCGGCTCTGGCGAGGCGG  
CTCAGGTGGCGGCGGCTCCGGCGGAGGTTTCGCTTCAGCTTCTGCCAGGTG  
GGCAATCACTCTGATTTCCGTCAACGGAATCTTCGTATCTGCTGCCTGAC  
CTACTGCTTTGCTCCCAGATGTAGAGAGCGCCGCCGCAACGAGCGCCTGC  
GTCGTGAGAGCGTGAGGCCTGTGTGAGAATTCCGCCCCCCCCCCCCCG  
GGCAGAGCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTC  
GGCAATTGATCCGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAA  
GTGATGTCTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGT  
ATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTTCGCAACGGGTTTGCCG  
CCAGAACACAGGCTTGATCACGCGTGCCACCATGCCCCCGCTAGACTAC  
TCTTTTTCTGCTTTTCTGACTCCGATGGAGGTGCGCCCCGAGGAGCCAT  
TAGTGGTGAAGGTGGAGGAAGGTGACAACGCCGTGTTACAGTGCCTGAAG  
GGAACCTCCGACGGCCCTACTCAGCAGCTGACCTGGTCTCGCGAGAGCCCC  
CCTGAAGCCGTTCTTGAAACTCAGTCTTGGCCTGCCCGGCCTAGCAATCCA  
CATGAGGCCTCTGGCGATTGGCTCTTTATTTTCAACGTGTCCAGCAGAT  
GGGCGGCTTCTATCTATGCCAGCCTGGCCCACCCTCTGAAAAGGCTTGCCA  
ACCAGGTTGGACTGTCAACGTGGAGGGCAGCGGGGAGCTGTTACAGGTGGA  
ACGTGTCCGACCTGGGCGGTCTCGGGTGCGGGCTTAAAAACCGCAGCTCG  
GAGGGGCCCCAGCTCCCCATCCGGTAAGCTCATGTCCCCTAAGCTGTACGTC  
TGGGCCAAGGACCGCCCTGAGATCTGGGAAGGAGAGCCTCCGTGCGTGCC  
CCCGCGTGATTGCTCAACCAGAGCCTGTCCCAGGACCTCACGATGGCAC  
CCGGTAGCACCCCTGTGGTTATCCTGCGGCGTCCCCCCCCGACTCCGTGTCCC  
GTGGACCCCTGAGTTGGACACATGTCCACCCCAAGGGCCCTAAGTCATTG  
TTGCTTTTGGAGCTGAAAGATGACCGGCCGGCTCGTGACATGTGGGTGAT  
GGAGACCGGCCTGCTGCTGCCGCGCGCCACGGCCCAGGATGCGGGGAAGT  
ACTACTGTCAACGCGGCAACCTGACCATGTCTGTTCCACTTGGAGATCACCG  
CGCGCCCAGTGCTGTGGCATTGGCTTCTCCGAACCGGCGGATGGAAGGTT  
TCCGCTGTCACCCCTGGCCTACCTGATCTTTTGTCTGTGCTCCCTGGTTGGTA  
TCCTGCACCTGCAGCGCGCTCTGGTACTGCGCCGGAAGCGC<sub>taa</sub>GTTTGT  
GAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAACA  
CTTGCTGGGATTACTTCTTCAGGTAAACCAACAGAAGGCTCGAGAAGGT  
ATATTGCTGTTGACAGTGAGCGCGCAAGCTGACCCTGAAGTTCATTAGTG  
AAGCCACAGATGTAATGAACCTCAGGGTCAGCTTGCTTGCTACTGCCTCG  
GAATTCAAGGGGCTACTTTAGGAGCAATTATCTTGTTTACTAAACTGAAT  
ACCTTGCTATCTCTTTGATACATTTTTACAAAGCTGAATTAAATGTTATA  
AATTAAATCACTTTTTTTGA (SEQ ID NO:151)

|    |                                                       |
|----|-------------------------------------------------------|
|    |                                                       |
| N  | AGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAGGAAAACT       |
| F  | GTTTCATACAGAAGGCGTCAATTAGGAGGAAAACTGTTTCATACAGAAG     |
| A  | GCGTCAATTGGTCCCATCGAATTAGGAGGAAAACTGTTTCATACAGAAG     |
| T- | GCGTCAATTAGGAGGAAAACTGTTTCATACAGAAGGCGTCAATTAGGAG     |
| Te | GAAAACTGTTTCATACAGAAGGCGTCAATTGGTCCCGGGACATTTTGAC     |
| IL | ACCCCCATAATATTTTCCAGAAATTAACAGTATAAATTGCATCTCTTGTTT   |
| 12 | AAGAGTTCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCC    |
| -  | TGGACCGGTTCTAGAGCGCTTTAATTAAGCCACCATGGATTGGACCTGGAT   |
| E  | TCTGTTCCCTCGTGGCCGCCGCTACCCGCGTGCACCTCCATCTGGGAGCTGAA |
| F1 | GAAAGACGTGTACGTGGTGGGAATTGGATTGGTACCCCGACGCCCCAGGCG   |
| a- | AGATGGTGGTTCTGACTTGCGACACGCCGGAGGAGGACGGCATCACCTGG    |
| Fa | ACGCTGGACCAGAGCAGCGAAGTGCTGGGCTCTGGTAAGACCTTAACCAT    |
| s- | CCAGGTCAAGGAGTTCGGTGATGCAGGACAGTACACGTGTCACAAGGGCG    |
| D  | GGGAGGTCTTGTTCCCATAGCCTGCTGCTCCTGCACAAGAAGGAAGACGGC   |
| N  | ATCTGGTCTACGGATATCCTTAAAGACCAGAAGGAGCCCAAGAACAAAAC    |
|    | CTTCCTGCGATGCGAGGCTAAGAATTACTCCGGGCGCTTTACCTGTTGGTG   |
|    | GCTCACAAACCATCTCGACCGACCTGACCTTCTCCGTGAAGTCTTCCAGGGG  |
|    | ATCTTCTGATCCGCAAGGCGTTACCTGCGGGGCCGCTACCTGTGCGGTGA    |
|    | GCGCGTCCGTGGTGACAACAAGGAGTACGAGTACTCGGTGGAATGCCAGG    |
|    | AGGACAGCGCCTGCCCAGCGGCCGAAGAGTCCCTTGCCCATCGAGGTGATG   |
|    | GTGGACGCTGTACACAAGCTCAAGTACGAGAACTACACCTCCTCCTTCTTT   |
|    | ATACGCGACATCATCAAGCCTGACCCCCCTAAAAACCTGCAGCTGAAGCC    |
|    | CCTTAAAAACTCTCGACAGGTGAGGTATCCTGGGAGTACCCCGACACCT     |
|    | GGTCTACTCCTCATTTCGTATTTACGCTGACCTTCTGCGTGCAGGTGCAGG   |
|    | GCAAGTCCAAGCGCGAGAAGAAGGACCGCGTGTTACCGACAAAACAG       |
|    | CGCGACTGTCATTTGTCGGAAGAACGCGAGCATCTCCGTACGGGCACAGG    |
|    | ATCGCTATTACTCCAGCAGTTGGTCAGAGTGGGCTTCGGTTCCGTGTTCTG   |
|    | GAGGAGGTGGCGGCGGCTCACGCAACCTGCCCGTGGCGACGCCGGACCCG    |
|    | GGTATGTTTCTTGTCTGCACCACTCTCAGAACTTGCTCCGTGCCGTCTCG    |
|    | AATATGCTGCAGAAGGCCCCGACACTCTGGAGTTCTATCCATGCACTAG     |
|    | TGAGGAGATCGATCATGAGGACATCACCAAGGACAAGACATCCACCGTGG    |
|    | AGGCTTGTCTGCCGCTGGAGTTGACAAAGAACGAGAGCTGCCTCAATAGC    |
|    | CGCGAGACTTCCTTCATCACCAACGGCTCCTGCCTCGCGAGCCGTAAGACC   |
|    | TCCTTTATGATGGCGCTGTGCCTGTCAAGTATCTACGAAGATCTGAAAATG   |
|    | TATCAGGTGGAGTTCAAAACATATGAACGCAAACTCCTGATGGATCCCAA    |
|    | GCGCCAAATTTTCTTGACCAAGAACATGCTTGCCGTGATCGACGAGCTGAT   |
|    | GCAGGCCCTGAACCTCAACTCCGAGACCGTCCCTCAGAAGTCGTCTCTGG    |
|    | AGGAACCCGACTTTTACAAGACAAAGATCAAGCTGTGCATCCTGCTACAC    |
|    | GCGTTCCGCATTCGGGCCGTGACCAATTGACCGCGTGATGTCCTACCTGAAT  |
|    | GCCTCGTCCGGCGGGGGGGGCTCCGGTGGGGGCGGCTCTGGCGGAGGCGG    |
|    | CTCAGGTGGCGGCGGCTCCGGCGGAGGTTTCGCTTCAGCTTCTGCCAGTTG   |
|    | GGCAATCACTCTGATTTCCGTCAACGGAATCTTCGTATCTGCTGCCTGAC    |
|    | CTACTGCTTTGCTCCCAGATGTAGAGAGCGCCGCCGCAACGAGCGCCTGC    |
|    | GTCGTGAGAGCGTGAGGCCTGTGTGAGAATTCCGCCCCCCCCCCCCCGG     |
|    | GGCAGAGCGCACATCGCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTC      |
|    | GGCAATTGATCCGGTGCCTAGAGAAGGTGGCGCGGGGTAACTGGGAAA      |
|    | GTGATGTCGTGTACTGGCTCCGCCTTTTCCCAGGGGTGGGGGAGAACCGT    |

|  |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
|--|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|  | ATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTTCGCAACGGGTTTGCCG<br>CCAGAACACAGGCTTGATCACGCGTGCCACCATTGGATTGGACCTGGATCC<br>TGTTCTGGTGGCTGCAGCCACCCGTGTTTCATTCCCAGGTCACCGACATCA<br>ACAGCAAGGGCCTGGAGCTGCGCAAGACCGTGACGACCGTGGAGACCCA<br>GAACCTGGAGGGTTTGCACCATGACGGCCAGTTCTGTCACAAGCCGTGCC<br>CACCCGGGGAGCGCAAGGCCCGGGACTGCACTGTCAACGGGGACGAGCC<br>CGACTGCGTGCCTTGTCAGGAGGGAAAAGAGTACACGGACAAAGCGCACT<br>TTTCTTCCAAGTGCCGAAGGTGTGCGCTGTGCGACGAGGGCCACGGTCTCG<br>AGGTGGAAATTAAGTGCCTCGCACCCAGAACACTAAGTGCCGTTGCAAG<br>CCCAACTTCTTCTGTAATTCGACCGTATGCGAGCACTGTGATCCTTGCACA<br>AAATGTGAGCACGGCATCATCAAGGAATGTACGTTGA (SEQ ID NO:152) |
|--|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

[00297]

**IV. Recombinant Expression Vectors, Lentiviral Expression Systems, Packaging Cell Line**

[00298] In an embodiment of the invention, the inventive nucleic acid molecule disclosed herein is carried in a recombinant expression vector. Accordingly, an embodiment of the invention provides a recombinant expression vector comprising any of the inventive nucleic acid molecules described herein with respect to other aspects of the invention.

[00299] In some embodiments, the nucleotide sequences encoding the one or more GOIs, e.g., cytokines selected from the group consisting of IL-12, IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof, are provided by two or more recombinant expression vectors. For example, provided herein are a recombinant expression vector comprising a nucleotide sequence encoding IL-12 or a variant thereof; and a second recombinant expression vector comprising a nucleotide sequence encoding IL-15 or a variant thereof. In some embodiments, provided herein are a recombinant expression vector comprising a nucleotide sequence encoding IL-12 or a variant thereof; and a second recombinant expression vector comprising a nucleotide sequence encoding IL-2 or a variant thereof.

[00300] For purposes herein, the term "recombinant expression vector" means a genetically-modified oligonucleotide or polynucleotide construct that permits the expression of an mRNA, protein, polypeptide, or peptide by a host cell, when the construct comprises a nucleotide sequence encoding the mRNA, protein, polypeptide, or peptide, and the vector is contacted with the cell under conditions sufficient to have the mRNA, protein, polypeptide, or peptide expressed within the cell. The vectors of the invention are not naturally-occurring as a whole. However, parts of the vectors can be naturally-occurring. The recombinant

expression vector can comprise any type of nucleotides, including, but not limited to DNA and RNA, which can be single-stranded or double-stranded, synthesized or obtained in part from natural sources, and which can contain natural, non-natural or altered nucleotides. The recombinant expression vectors can comprise naturally-occurring or non-naturally-occurring internucleotide linkages, or both types of linkages. Preferably, the non-naturally occurring or altered nucleotides or internucleotide linkages do not hinder the transcription or replication of the vector. The vector may contain regulatory nucleic acid sequences which provide for expression of the inventive nucleic acid.

**[00301]** The recombinant expression vector can be any suitable recombinant expression vector. Suitable vectors include those designed for propagation and expansion or for expression or both, such as plasmids and viruses. For example, the vector can be selected from the pUC series (Fermentas Life Sciences, Glen Burnie, MD), the pBluescript series (Stratagene, LaJolla, CA), the pET series (Novagen, Madison, WI), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clontech, Palo Alto, CA).

**[00302]** Bacteriophage vectors, such as  $\lambda$ GT10,  $\lambda$ GT11,  $\lambda$ Zap II (Stratagene),  $\lambda$ EMBL4, and  $\lambda$ NMI 149, also can be used. Examples of plant expression vectors useful in the context of the invention include pBI01, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clontech). Examples of animal expression vectors useful in the context of the invention include pEUK-Cl, pMAM, and pMAMneo (Clontech).

**[00303]** In some embodiments, the recombinant expression vector is a viral vector. Suitable viral vectors include, without limitation, lentiviral, retroviral, alphaviral, vaccinia, adenoviral, adenoassociated viral, herpes viral, and fowl pox viral vectors, and preferably have a native or engineered capacity to transform T cells.

**[00304]** The recombinant expression vectors can be prepared using standard recombinant DNA techniques described in, for example, Green and Sambrook, *Molecular Cloning: A Laboratory Manual*, (4th Ed.) Cold Spring Harbor Laboratory Press, New York (2012). Constructs of expression vectors, which are circular or linear, can be prepared to contain a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived, e.g., from ColEI, 2  $\mu$  plasmid,  $\lambda$ , SV40, bovine papilloma virus, and the like.

**[00305]** The recombinant expression vector can comprise regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the

type of host (e.g., bacterium, fungus, plant, or animal) into which the vector is to be introduced, as appropriate, and taking into consideration whether the vector is DNA- or RNA-based.

[00306] The recombinant expression vector can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to antibiotics, heavy metals, etc., complementation in an auxotrophic host to provide prototrophy, and the like. Suitable marker genes for the recombinant expression vectors include, for instance, neomycin/G418 resistance genes, hygromycin resistance genes, histidinol resistance genes, tetracycline resistance genes, and ampicillin resistance genes.

[00307] The recombinant expression vector can comprise a native or normative promoter operably linked to the nucleic acid molecule. Preferably, the promoter is functional in T cells. The selection of a promoter, e.g., strong, weak, inducible, tissue-specific and developmental-specific, is within the ordinary skill of the artisan. Similarly, the combining of a nucleotide sequence with a promoter is also within the skill of the artisan. The promoter can be a non-viral promoter or a viral promoter, e.g., an NFAT promoter, a cytomegalovirus (CMV) promoter, an SV40 promoter, an RSV promoter, or a promoter found in the long-terminal repeat of the murine stem cell virus.

[00308] Further provided herein is a lentiviral expression system comprising a transfer vector comprising the nucleic acid molecules disclosed herein which can be transcribed into a recombinant lentiviral RNA molecule for packaging into a lentiviral particle, and one or more helper vectors or helper plasmids encoding an Env protein, a Gag protein, a Pol protein, and a Rev protein. In some embodiments, the Env protein is selected from the group consisting of Ba-EVTR, VSV-G, and RD114.

[00309] In some embodiments, the recombinant lentiviral RNA molecule may comprise at least a portion of a lentivirus genome, including 5' and 3' LTRs. In some embodiments, the lentivirus genome may be modified to inhibit replication and limit pathogenicity, while retaining function. For example, the *env* gene, the *gag* gene, the *pol* gene, and the *rev* gene may be removed from the lentivirus genome and included in helper plasmids. In some embodiments, the recombinant lentiviral RNA molecule is comprised by a lentiviral vector or recombinant lentiviral particle as described elsewhere in this disclosure.

**[00310]** In some embodiments, two helper vectors or helper plasmids encode an Env protein, a Gag protein, a Pol protein, and a Rev protein. In some embodiments, three helper vectors or helper plasmids encode an Env protein, a Gag protein, a Pol protein, and a Rev protein. In some embodiments, four helper vectors or helper plasmids encode an Env protein, a Gag protein, a Pol protein, and a Rev protein. Any combination of helper vectors or helper plasmids can be used to encode an Env protein, a Gag protein, a Pol protein, and a Rev protein. For example, in some embodiments, one helper vectors or helper plasmids encode an Env protein, a Gag protein, and a Pol protein. In some embodiments, one helper vectors or helper plasmids encode a Gag protein, a Pol protein, and a Rev protein. In some embodiments, one helper vectors or helper plasmids encode a Gag protein, and a Pol protein. In some embodiments, one helper vectors or helper plasmids encode an Env protein, and a Rev protein.

**[00311]** A packaging cell line can be used to package a nucleic acid, e.g., an RNA encoding a transgene, into a lentiviral vector. Accordingly, the systems and methods described herein may comprise, e.g., a lentiviral packaging cell line comprising at least one plasmid adapted for the production of a lentiviral vector, e.g., a lentiviral vector optionally comprising a transgene. Various lentiviral components useful for the production of a lentiviral vector are known in the art. See for example Zufferey et al., 1997, Nat. Biotechnol. 15:871-875 and Dull et al, 1998, J. Virol. 72(11): 8463 -8471. The different functions suitable for the production of a lentiviral vector can be provided to the packaging cells in a lentiviral packaging system comprising one or more nucleic acids (e.g., plasmids), e.g., at least one, two, three, or four plasmids, wherein one plasmid encodes a retroviral envelope protein (Env plasmid), one plasmid encodes one or more retroviral packaging proteins, e.g., Gag and Pol proteins (packaging plasmid or Gag-Pol plasmid), one plasmid encodes a lentiviral Rev protein (Rev plasmid) and one or more plasmids comprising at least one gene of interest (GOI) expression cassette (transfer vector). In some embodiments, the lentiviral packaging system further comprises, or a method described herein comprises use of, at least one, two, three, or four plasmids. In some embodiments, the lentiviral packaging system further comprises, or a method described herein comprises use of, a fifth plasmid. In certain embodiments, a method described herein comprises transfecting five plasmids into the packaging cell, wherein the fifth plasmid does not encode a protein of the lentiviral vector packaging system. In some embodiments, the lentiviral packaging system comprises one or more nucleic acids (e.g., plasmids), e.g., five plasmids, wherein one plasmid encodes an

expression vector, one plasmid encodes a Tat (e.g., pcDNATat), one plasmid encodes a Rev protein (e.g., pHCMV-Rev), one plasmid encodes a gagpol (e.g., pHCMV-gagpol), and one plasmid encodes VSV-G (e.g., pVSVG), e.g., as described in Rout-Pitt et al., J Biol. Methods 5(2): 1-9, 2018). In some embodiments, a plasmid may comprise a dual gene expression cassette, e.g., a bicistronic cassette, e.g., a bicistronic construct encoding two transgenes of interest. In some embodiments, the first transgene of interest encodes a first cytokine, e.g., TeIL-12, and the second transgene of interest encodes a second cytokine, e.g., IL-15. In some embodiments the retroviral packaging proteins are derived from a lentivirus, e.g., lentiviral packaging proteins, e.g., lentiviral gag and pol proteins.

**[00312]** In some embodiments, the lentiviral gag protein is a wild-type lentiviral gag protein, and in other embodiments it has one or more sequence modifications relative to the wild-type sequence. In some embodiments, the lentiviral pol protein is a wild-type lentiviral pol protein, and in other embodiments it has one or more sequence modifications relative to the wild-type sequence. In some embodiments, the rev protein is a wild-type rev protein, and in other embodiments it has one or more sequence modifications relative to the wild-type sequence. In some embodiments, the lentiviral vector packaging system may be a pseudotyped lentiviral vector packaging system, comprising a modified envelope protein, e.g., an envelope protein derived from a different virus or a chimeric envelope protein, e.g., the Env plasmid may encode a Ba-EVTR Env protein.

**[00313]** In some embodiments, a lentiviral vector is generated using a packaging system comprising pMDLgpRRE, pRSV-Rev and pMD.G plasmids (Dull et al., supra), but using a kanamycin resistance marker, e.g., a marker that confers resistance to both kanamycin and neomycin, e.g., neomycin phosphotransferase II instead of an ampicillin gene.

**[00314]** Therefore, further provided here is a packaging cell line comprising a recombination expression vector disclosed herein, for example, a transfer vector, comprising the nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12) and optionally a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof. In some embodiments, the cytokine is a tethered cytokine. In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a TeIL-12 and a tethered IL-15 (TeIL-15). In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a TeIL-12 and a tethered IL-18 (TeIL-18).

[00315] In some embodiments, the nucleic acid molecule further comprises a nucleic acid sequence encoding an shRNA. In some embodiments, the shRNA inhibits the expression of an immune checkpoint gene. In some embodiments, the shRNA inhibits the expression of PD-1.

[00316] Non-limiting examples of immune checkpoint genes that may be silenced or inhibited by the shRNA include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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 the shRNA may be selected from the group comprising PD-1, CTLA-4, LAG-3, TIM-3, Cish, CBL-B, TIGIT, TET2, TGF $\beta$ , 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 the shRNA may be selected from the group comprising PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, TET2, CISH, TGF $\beta$ R2, PRA, CBLB, BAFF (BR3), and combinations thereof.

[00317] In some embodiments, the packaging cell line is a human cell line. In some embodiments, the packaging cell line is a 293T cell line.

[00318] In some embodiments, the packaging cell line is a stable packaging cell line produced by the EuLV® system (Shenzhen Eureka Biotechnology Co., Limited, Shenzhen, China) described in Xue et al., *Cell & Gene Therapy Insights* **2022**; 8(2), 199–209, the content of which is incorporated by reference in its entirety.

[00319] Any suitable protocols known in the art may be used in developing the stable packaging cell line. For example, Broussau et al., *Mol. Thera.*, **2008**, 16:500-507 describes an inducible packaging cell line, 293SF-PacLV, for production of lentiviral vectors in serum-free culture, the content of which is hereby incorporated by reference in its entirety. A cell line derived from 293SF cells, expressing the repressor (CymR) of the cumate switch and the reverse transactivator (rtTA2S-M2) of the tetracycline (Tet) switch, is established first. Clones stably expressing the Gag/Pol and Rev genes of human immunodeficiency virus-1, and the glycoprotein of vesicular stomatitis virus (VSV-G), are generated next. Expression of



Rev and VSV-G is tightly regulated by the cumate and Tet switches in the 293SF-PacLV cell line. Two approaches are used to generate the packaging cell lines. In the first approach (Two-step), a stable clone expressing the Gag/Pol and Rev genes is first generated and characterized before producing a clone expressing VSV-G and additional Rev. In the second approach (One-shot), all the LV components (Gag/Pol, rev, and VSV-G) are added simultaneously through a single transfection event.

**[00320]** Two-step approach: A cell line derived from a clone of 293 cells (293SF) adapted to grow in suspension and in serum-free medium that expresses CymR is generated. 293SF were transfected with pMPGBFP/CMV5-CymR/tk-neo and a resistant pool of cells is isolated in the presence of neomycin. Clones are then isolated by limiting dilution of the pool. The clones are maintained for 6 weeks in the absence of selective pressure in order to test their stability. The CymR function is analyzed using an adenoviral vector (AdV) expressing  $\beta$ -galactosidase ( $\beta$ -gal) regulated by the CMV5-CuO promoter. The best  $\beta$ -gal On/Off ratio in the presence and absence of cumate is used in order to select the clone producing the optimal quantity of CymR. Clone G, which showed an On/Off ratio of 14, is selected as a recipient for the rtTA2<sup>S</sup>-M2 transactivator. 293SF-CymR-G cells are transfected with plasmid pUDHrtTA2<sup>S</sup>-M2.hydro. A pool of hygromycin-resistant cells is generated, and clones are obtained by limiting dilution of the pool in the absence of selection. The functioning of CymR and rtTA2<sup>S</sup>-M2 produced by the clones is tested using an AdV encoding the green fluorescent protein (GFP) regulated by the TR5 and CuO promoters. The clone with the best On/Off ratio is chosen for the development of the packaging cell lines.

**[00321]** This is done by transfecting it with pMPG-RSV-Rev/CMV-Gag/polRRE, a plasmid that encodes the Rev, Gag, and pol genes of human immunodeficiency virus-1, as well as the resistance for phleomycin as a fusion protein with GFP. Phleomycin-resistant clones are isolated in 96-well plates. The clones with the best GFP expression level are analyzed for LV production by transient transfection with pCSII-CMV5-GFPq and VSV-G. The titers obtained with the best clones are 10- to 55-fold lower than those obtained when the same clones are transfected with plasmids encoding Rev regulated by Rous sarcoma virus (RSV) (pRSV-Rev). Two clones (#19 and #64) are then subcloned by limiting dilution and analyzed for LV production by transient transfection. As observed for the parent clones, the LV titer obtained is significantly higher in the presence of additional Rev.

**[00322]** Because the previous results indicate that the quantity of Rev produced by the 293SF-Rev-Gag-Pol cells is not optimal, two of the best subclones (#19-17 and #64-8) are

co-transfected with plasmids encoding the puromycin resistance (pPuro), Rev (pkCMV5-CuO-Rev) and VSV-G (pTR5-CuO-VSVg-IRES-GFPq) to generate a cell line expressing VSV-G and more Rev. VSV-G is regulated by the cumate switch and the Tet switch, whereas Rev is regulated by the cumate switch only. The puromycin-resistant clones are first screened for VSV-G expression by measuring GFP (through the IRES-GFP) after induction with Dox and cumate. The LV production is then analyzed by transient transfection with pCSII-CMV5-GFPq. Co-transfection of the best clones with pRSV-Rev increases the LV titer by only twofold to fourfold, which is lower than the earlier level. The three best clones are subcloned in the absence of selective pressure. LV production is analyzed by transient transfection as described earlier. The production from the six best subclones can be improved by only twofold to threefold after the addition of Rev, thereby indicating that the quantity of Rev is nearly optimal. The efficacy of the double switch is investigated by measuring the increase of GFP expression (from the stably integrated VSV-G-IRES-GFPq cassette) after induction of clone #16-22. An induction factor over 2,500 is observed in the presence of cumate and Dox. GFP expression in the presence of the two inducers is much higher than when each inducer is used separately. In the presence of cumate only, the GFP expression level increases by a factor of 4.5, thereby indicating that the cumate switch improves the tightness of the Tet switch by this factor.

**[00323]** One-shot approach: Because Rev is required for the efficient expression of the protease from the Gag gene, which has been reported to be cytotoxic, even a relatively small amount of Rev could be detrimental to the cells. For this reason, a packaging cell line expressing Rev under very tight regulation is generated. pTR5-CuO-Rev, which contains the Rev coding sequence doubly regulated by the Tet switch and the cumate switch is constructed. The 293SF-CymR-rtTA2<sup>S</sup>-M2 cell line is co-transfected with plasmids encoding Rev, Gag, Pol, VSV-G, and the resistance for puromycin. Stable clones are selected in the presence of puromycin. Clones having the highest GFP induction factor after addition of Dox and cumate are analyzed for LV production by transfection with transfer vector pCSII-CMV5-GFPq. The two best clones are then subcloned without selection and analyzed for LV production. The transfection of the best subclones with pCSII-CMV5-GFPq and pRSV-Rev does not increase the amount of LVs produced, thereby suggesting that the quantity of Rev produced by the clones is not limiting.

**[00324]** The LV production levels obtained in the best clones when using the Two-step (#16-22) and One-shot (#29-6) approaches is then compared. Titers of  $8.6 \times 10^6$  and  $2.6 \times 10^7$

TU/ml are obtained for #16-22 and #29-6, respectively. The amount of p24 produced after induction is also evaluated by enzyme-linked immunosorbent assay (ELISA). The amounts of p24 produced are 39 and 571 ng/ml by #16-22 and #29-6, respectively. The fact that the specific activity (TU/ng of p24) is 219,000 for #16-22 and 45,000 for #29-6 indicates that the latter clone produced more defective or empty virions.

**[00325]** Briefly, Ba-EVTR, gag/pol, and rev are stably inserted into 293T cells to obtain packaging cell populations, and the optimal packaging cell line is obtained through monoclonal and titer screening. Then, a transfer vector comprising the nucleic acid molecules disclosed herein, i.e., a transfer vector encoding the GOI, for example, a transfer vector comprising the nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12) and optionally a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNF $\alpha$ , IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof, is integrated into the optimal packaging cell line. In some embodiments, the cytokine is a tethered cytokine. In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a TeIL-12 and a tethered IL-15 (TeIL-15). In some embodiments, the nucleic acid molecule comprising a nucleotide sequence encoding a TeIL-12 and a tethered IL-18 (TeIL-18).

**[00326]** In some embodiments, the nucleic acid molecule further comprises a nucleic acid sequence encoding an shRNA. In some embodiments, the shRNA inhibits the expression of an immune checkpoint gene. In some embodiments, the shRNA inhibits the expression of PD-1.

**[00327]** Non-limiting examples of immune checkpoint genes that may be silenced or inhibited by the shRNA include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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 the shRNA may be selected from the group comprising PD-1, CTLA-4, LAG-3, TIM-3, Cish, CBL-B, TIGIT, TET2, TGF $\beta$ , 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 the shRNA

may be selected from the group comprising PD-1, LAG-3, TIM-3, CTLA-4, TIGIT, TET2, CISH, TGF $\beta$ R2, PRA, CBLB, BAFF (BR3), and combinations thereof.

**[00328]** In some embodiments, the packaging cell line comprises a recombinant DNA encoding an Env protein, a Gag protein, a Pol protein, and a Rev protein. In some embodiments, the recombinant DNA encoding one or more of the Env protein, the Gag protein, the Pol protein, and the Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Env protein, the Gag protein, and the Pol protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Env protein, the Gag protein, and the Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Env protein, the Pol protein, and the Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Gag protein, the Pol protein, and the Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the Env protein is selected from the group consisting of Ba-EVTR, VSV-G, and RD114. In some embodiments, the Env protein is a Ba-EVTR Env protein. In some embodiments, the recombinant DNA encoding one or more of the Env protein, the Gag protein, the Pol protein, and the Ba-EVTR Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Ba-EVTR Env protein, the Gag protein, and the Pol protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Ba-EVTR Env protein, the Gag protein, and the Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the recombinant DNA encoding the Ba-EVTR Env protein, the Pol protein, and the Rev protein is integrated into the genome of the packaging cell line.

**[00329]** In some embodiments, the recombinant DNA encoding the Env protein, the Gag protein, the Pol protein, and the Rev protein is integrated into the genome of the packaging cell line. In some embodiments, the nucleic acid molecule disclosed herein, e.g., the recombinant lentiviral proviral DNA molecule, is integrated into the genome of the packaging cell line.

**[00330]** In some embodiments, the different functions for production of a lentiviral vector are provided to a plurality of host cells, e.g., mammalian cells, e.g., HEK293 cells, e.g., Expi293F cells (e.g., plurality of Expi293F cells growing in suspension under serum-free conditions) by transfection, e.g., transient or stable transfection, of a lentiviral packaging

system adapted for producing lentiviral vectors. In some embodiments, at least 25%, at least 30%, at least 35%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% of host cells, e.g., HEK293 cells, e.g., Expi293F cells are transfected. Methods for transfection or infection are well known by those of skill in the art. In some embodiments, at least 0.3pg, at least 0.4pg, at least 0.5pg, at least 0.6pg, at least 0.7pg, at least 0.8pg cells, at least 0.9pg, or at least 1.0 pg of lentiviral packaging system is provided per million cells for transfection. In some embodiments, a transfection reagent is used for transfecting the host cells, e.g., mammalian cells, e.g., HEK293 cells, e.g., Expi293F cells. In some embodiments, a transfection reagent is used. Transfection reagents are well known in the art and are available from commercial suppliers. Examples of transfection reagents include but are not limited to, Lipofectamine™ (Invitrogen), Polifectamine, LentiTran (Origene), PEIpro® (Polyplus), FectoVIR® - AAV (Polyplus), and ProFection® (Promega).

#### **V. Recombinant Lentiviral Particle And Method Of Making**

[00331] This disclosure provides, in some embodiments, method of making a recombinant lentiviral particle. The following general steps may be used. First, culturing a population of packaging cells in a cell culture medium. Once sufficient numbers of packaging cells are obtained, the desired nucleic acids, such as a lentiviral vector comprising a nucleic acid molecule encoding the cytokines disclosed herein, can be introduced into the packaging cells. Additional nucleic acids that may be introduced into the packaging cells include plasmids that promote packaging, e.g., plasmids encoding viral gag, pol, env, and rev. The packaging cells then begin to produce recombinant lentiviral particles. After transfection, a nuclease such as benzonase may be added to the culture media.

[00332] In some embodiments, a stable packaging cell line with the VSV-G, gag/pol, and rev integrated into the genome can be used for the production of the recombinant lentiviral particles. In some embodiments, the stable packaging cell line further comprises the lentiviral vector comprising a nucleic acid molecule encoding the cytokines disclosed herein integrated into the genome.

[00333] Any suitable protocols known in the art may be used for producing the recombinant lentiviral particles using a stable packaging cell line. For example, Broussau et al., supra, describes lentiviral particle production using a stable packaging cell line, the content of which is hereby incorporated by reference in its entirety.

[00334] Neither the preparation of large quantities of plasmid nor the transfection procedure would be necessary if a cell line (a producer) that synthesizes all the essential viral functions including the viral RNA are available. Using such a cell line, LV production can be initiated solely by adding the inducers, such as Dox and cumate. The ability of packaging cells to generate stable producers is evaluated. Clones #29-6 and #16-22 are transduced with a conditional-SIN-LV, produced by transfecting packaging cells with the transfer vector pLVR2-GFP23. Clones are isolated by limiting dilution of the pool of transduced cells. The clones having the highest GFP expression following induction with Dox are expended. The production in serum-free suspension culture of the best clones from parent cells #16-22 (16-22-22) and #29-6 (#29-6-14) is tested. The medium is changed on a daily basis and the number of infectious particles is determined by flow cytometry.

[00335] The amount of p24 produced is also analyzed by ELISA. The behavior of the two clones is quite similar, except for the absolute amount of LVs produced, which is two to three times higher for clones #29-6-14. The number of infectious particles produced increases every day until it reaches a maximum at day 4. It then decreases progressively. The highest titer ( $3.4 \times 10^7$  TU/ml) is obtained at day 4 when #29-6-14 is used. The quantity of p24 produced by the cells at different time points follows the same pattern. On day 1, the specific activity of the viral preparation (TU/ng of p24) is 54,000 and 166,000 for #16-22-22 and #29-6-14, respectively, and decreases progressively. At the end of the production (day 6 or 7) the specific activity is lower by a factor of 3 to 4, thereby indicating that more defective particles are produced at later time points.

[00336] The relative stability of four different producer clones derived from packaging cells #29-6 is evaluated. The LV production is compared after 4 weeks and 18 weeks of culture in the absence of selective pressure. The titers obtained with these four clones does not decrease significantly after 18 weeks of culture. These data indicate that, using the packaging cells described in this study, it is possible to obtain producers whose long-term stability is more than adequate for a large-scale production process. In addition, tests to detect the presence of replication-competent lentiviruses are carried out by infecting 293 cells using concentrated stock of LVs. No replication-competent lentiviruses are detected by ELISA analysis for P24, and no VSV-G is detected by polymerase chain reaction (PCR).

[00337] Because subcloning and analysis of individual clones is a time-consuming process, whether high levels of LVs can be obtained using a pool of cells is investigated. For this experiment, packaging cells #29-6 are transduced with a conditional-SIN-LV produced

by transfecting packaging cells with pTet07-CSII-CMVGFPq. The production efficiency of the pool of producers is then tested in serum-free suspension culture in shake flasks. The medium is changed on a daily basis and the number of infectious particles is determined by flow cytometry. The production pattern of the pool is very similar to that of the two clones, except for the fact that the highest titer ( $1.9 \times 10^7$  TU/ml) is obtained at day 3 instead of day 4, and that the duration of useful production is one day shorter.

[00338] Without wishing to be bound by theory, in some embodiments, the cell culture medium is a source of contaminating nucleic acids to the final lentiviral preparation, e.g., the culture medium may contain packaging cell DNA from lysed packaging cells. Accordingly, addition of benzonase to the cell culture medium may degrade the contaminating nucleic acids, allowing for improved purification of the recombinant lentiviral particles.

[00339] Next, recombinant lentiviral particles can be harvested from the packaging cell culture to begin purification of the recombinant lentiviral particles. In some embodiments, harvesting of recombinant lentiviral particles comprises separating the supernatant or cell culture media from the packaging cell. In some embodiments, the packaging cell is not lysed before clarification. In some embodiments, the packaging cells may be lysed, and the lysate may be clarified.

[00340] Naturally occurring lentiviruses are a genus of viruses of the Retroviridae family, characterized by a long incubation period. Lentiviruses can typically deliver a significant amount of genetic information into the DNA of the host cell. Examples of lentiviruses include HIV (human immunodeficiency vims; including HIV type 1, and HIV type 2), the etiologic agent of the human acquired immunodeficiency syndrome (AIDS); visna-maedi, which causes encephalitis (visna) or pneumonia (maedi) in sheep, the caprine arthritis-encephalitis vims, which causes immune deficiency, arthritis, and encephalopathy in goats; equine infectious anemia vims, which causes autoimmune hemolytic anemia, and encephalopathy in horses; feline immunodeficiency vims (FIV), which causes immune deficiency in cats; bovine immune deficiency vims (BIV), which causes lymphadenopathy, lymphocytosis, and possibly central nervous system infection in cattle; and simian immunodeficiency vims (SIV), which cause immune deficiency and encephalopathy in sub-human primates. Diseases caused by these vimses are characterized by a long incubation period and protracted course. Usually, the viruses latently infect monocytes and macrophages, from which they spread to other cells. HIV, FIV, and SIV also readily infect T lymphocytes (i.e., T-cells).

[00341] Further provided herein is a recombinant lentiviral particle comprising the recombinant lentiviral RNA molecule disclosed herein. In some embodiments, the recombinant lentiviral particle is produced by the packaging cell line disclosed herein.

[00342] In some embodiments, the recombinant lentiviral particle comprises the recombinant lentiviral vector disclosed herein. In some embodiments, the recombinant lentiviral vector comprises the recombinant lentiviral RNA molecule disclosed herein. In some embodiments, the recombinant lentiviral particle further comprises a capsid enclosing the recombinant RNA viral genome. In some embodiments, the recombinant lentiviral particle further comprises an Env protein selected from the group consisting of Ba-EVTR, VSV-G, and RD114. In some embodiments, the recombinant lentiviral particle further comprises a reverse transcriptase.

Add section on making non-gene-edited TILs

## **VI. Method Of Making Gene-Edited TILs**

[00343] In some embodiments of the present invention directed to methods for expanding TIL populations, the methods comprise one or more steps of gene-editing at least a portion of the TILs in order to enhance their therapeutic effect. As used herein, “gene-editing,” “gene editing,” and “genome editing” refer to a type of genetic modification in which DNA is permanently modified in the genome of a cell. *e.g.*, DNA is inserted, deleted, modified or replaced within the cell’s genome. In some embodiments, gene-editing causes the expression of a DNA sequence to be silenced (sometimes referred to as a gene knockout) or inhibited/reduced (sometimes referred to as a gene knockdown). In other embodiments, gene-editing causes the expression of a DNA sequence to be enhanced (*e.g.*, by causing over-expression). In accordance with embodiments of the present invention, gene-editing technology is used to enhance the effectiveness of a therapeutic population of TILs.

[00344] In some embodiments of the present invention, provided herein is a method of making a population of gene-edited TILs, comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium; b) performing a second expansion by culturing the population of TILs in a second cell culture medium; and c) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs, wherein the population of gene-edited TILs expresses one or more cytokines, such as IL-12, IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-



21, IL-23, IL-27, IL-33, IFN gamma, TNF $\alpha$ , IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof.

[00345] Cell surface markers PD-1, CD39, and CD103 represent the leading biomarkers to identify tumor-specific T cells but these TILs are terminally differentiated. Up-regulation of CD137 (4-1BB; TNFSR9) on recently activated CD8<sup>+</sup> T-cells has been used to identify tumor antigen-specific T-cells (Draghi A., et al. *Front Immunol* **2021**;12:705422, the content of which is hereby incorporated by reference in its entirety) which are antigen specific and tend to have a fitter/younger phenotype (c-fos and c-jun expression). CD137 through costimulation effects provides antigen-primed T cells with augmented survival, proliferation and effector functions as well as metabolic advantages.

[00346] Therefore, the present invention provides a method which enriches tumor-specific CD8 TILs (preserving the breadth of the TCR tumor-reactive repertoire), while providing CD4 TILs in the same process. In some embodiments, the method comprises priming tumors and use of CD137 as a marker to enrich and expand tumor-specific T cells without knowledge of epitope specificities. See, e.g., Fig. 48.

#### A. Obtain Patient Tumor Sample

[0001] In general, TILs are initially obtained from a patient tumor sample ("primary TILs") and then expanded into a larger population for further manipulation as described herein.

[0002] A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In some embodiments, multilesional sampling is used. In some embodiments, surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells includes multilesional sampling (*i.e.*, obtaining samples from one or more tumor sites and/or locations in the patient, as well as one or more tumors in the same location or in close proximity). In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of 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 endometrial

cancer, cervical cancer, head and neck cancer (including, for example, head and neck squamous cell carcinoma (HNSCC)), glioblastoma (GBM), gastrointestinal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, and non-small cell lung carcinoma. In some embodiments, useful TILs are obtained from a melanoma.

**[0003]** Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 mm<sup>3</sup>, with from about 2-3 mm<sup>3</sup> being particularly useful. In some embodiments, the TILs are cultured from these fragments using enzymatic tumor digests. Such tumor digests may be produced by incubation in enzymatic media (*e.g.*, Roswell Park Memorial Institute (RPMI) 1640 buffer, 2 mM glutamate, 10 mcg/mL gentamicine, 30 units/mL of dNase and 1.0 mg/mL of collagenase) followed by mechanical dissociation (*e.g.*, using a tissue dissociator). Tumor digests may be produced by placing the tumor in enzymatic media and mechanically dissociating the tumor for approximately 1 minute, followed by incubation for 30 minutes at 37 °C in 5% CO<sub>2</sub>, 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.

#### **B. Tumor Fragmentation and/or Digest**

**[0004]** As indicated above, in some embodiments, the TILs are derived from solid tumors. In some embodiments, the solid tumors are not fragmented. In some embodiments, the solid tumors are not fragmented and are subjected to enzymatic digestion as whole tumors. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease for 1-2 hours. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease for 1-2 hours at 37°C, 5% CO<sub>2</sub>. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease for

1-2 hours at 37°C, 5% CO<sub>2</sub> with rotation. In some embodiments, the tumors are digested overnight with constant rotation. In some embodiments, the tumors are digested overnight at 37°C, 5% CO<sub>2</sub> with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[0005] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[0006] 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.

[0007] In some embodiments, the enzyme mixture comprises DNase. In some embodiments, the working stock for the DNase is a 10,000 IU/ml 10X working stock.

[0008] 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.

[0009] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml DNase, and 1 mg/ml hyaluronidase.

[0010] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml DNase, and 1 mg/ml hyaluronidase.

[0011] In some embodiments, the enzyme mixture comprises neutral protease. In some embodiments, the working stock for the neutral protease is reconstituted at a concentration of 175 DMC U/mL.

[0012] In some embodiments, the enzyme mixture comprises neutral protease, dNase, and collagenase.

[0013] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml dNase, and 0.31 DMC U/ml neutral protease. In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml dNase, and 0.31 DMC U/ml neutral protease.

[0014] In general, the harvested cell suspension is called a “primary cell population” or a “freshly harvested” cell population.

[0015] 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 prior to gene-editing.

**[0016]** In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained. In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 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  $27 \text{ mm}^3$ . In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about  $1300 \text{ mm}^3$  to about  $1500 \text{ mm}^3$ . In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about  $1350 \text{ mm}^3$ . 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. In some embodiments, the multiple fragments comprise about to about 100 fragments.

**[0017]** 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  $1 \text{ mm}^3$  and  $10 \text{ mm}^3$ . In some embodiments, the tumor fragment is between about  $1 \text{ mm}^3$  and  $8 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $1 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $2 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $3 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $4 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $5 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $6 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $7 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $8 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $9 \text{ mm}^3$ . In some embodiments, the tumor fragment

is about 10 mm<sup>3</sup>. In some embodiments, the tumors are 1-4 mm x 1-4 mm x 1-4 mm. In some embodiments, the tumors are 1 mm x 1 mm x 1 mm. In some embodiments, the tumors are 2 mm x 2 mm x 2 mm. In some embodiments, the tumors are 3 mm x 3 mm x 3 mm. In some embodiments, the tumors are 4 mm x 4 mm x 4 mm.

**[0018]** In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of fatty tissue on each piece.

**[0019]** In some embodiments, the tumor fragmentation is performed in order to maintain the tumor internal structure. In some embodiments, the tumor fragmentation is performed without preforming a sawing motion with a scalpel. In some embodiments, the TILs are obtained from tumor digests. In some embodiments, tumor digests were generated by incubation in enzyme media, for example but not limited to RPMI 1640, 2 mM GlutaMAX, 10 mg/mL gentamicin, 30 U/mL dNase, and 1.0 mg/mL collagenase, followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). After placing the tumor in enzyme media, the tumor can be mechanically dissociated for approximately 1 minute. The solution can then be incubated for 30 minutes at 37 °C in 5% CO<sub>2</sub> and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% CO<sub>2</sub>, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% CO<sub>2</sub>. 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.

**[0020]** In some embodiments, the harvested cell suspension prior to the first expansion step is called a “primary cell population” or a “freshly harvested” cell population.

**[0021]** In some embodiments, cells can be optionally frozen after sample harvest and stored frozen prior to entry into the expansion described in further detail below.

**[0022]** In some embodiments, the TILs are obtained by thawing a cryopreserved tumor digest comprising a first population of TILs from a tumor resected from a subject. In some embodiments, the first population of TILs is obtained from a tumor resected from a subject by processing a tumor sample obtained from the subject into a tumor digest. In some embodiments, the tumor digest or tumor fragments comprise a first population of TILs and are obtained from a tumor that was resected from the subject.

**[0023]** In some embodiments, the methods disclosed herein comprise adding a tumor digest or tumor fragments into a closed system, wherein the tumor digest or tumor fragments comprise a first population of TILs and are obtained from a tumor that was resected from the subject. In some embodiments, the methods disclosed herein comprise performing a first expansion by thawing a cryopreserved tumor digest comprising a first population of TILs from a tumor that was resected from the subject. In some embodiments, the methods disclosed herein comprise obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into a tumor digest or multiple tumor fragments.

**[0024]** In some embodiments, a tumor lysate can be further obtained from the tumor digest through several freeze-thaw cycles or mass spectrometry procedures.

**[0025]** 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 priming step.

**[0026]** In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

**[0027]** 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.

**[0028]** 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.

**[0029]** 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.

**[0030]** In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 1000 IU/mL DNase, and 1 mg/mL hyaluronidase.

[0031] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 500 IU/mL DNase, and 1 mg/mL hyaluronidase.

[0032] 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.

[0033] In some embodiments, the TILs are not obtained from tumor digests. In some embodiments, the solid tumor cores are not fragmented.

[0034] In some embodiments, obtaining the first population of TILs comprises a multilesional sampling method.

[0035] 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 (dispase), 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.

[0036] 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).

[0037] 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.

[0038] 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.

[0039] 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.

[0040] 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.

[0041] In some embodiments, the enzyme mixture includes about 10.2- $\mu$ L of neutral protease (0.36 DMC U/mL), 21.3- $\mu$ L of collagenase (1.2 PZ/mL) and 250- $\mu$ L of DNase I (200 U/mL) in about 4.7 mL of sterile HBSS.

### C. Priming

[0042] In some embodiments, the method comprises a priming step, wherein the tumor digest is primed in the presence of IFN $\gamma$  (see Fig. 48). In some embodiments, the IFN $\gamma$  is present at a concentration of about 10 ng/mL, about 20 ng/mL, about 30 ng/mL, about 40 ng/mL, about 50 ng/mL, about 100 ng/mL, about 200 ng/mL, about 300 ng/mL, about 400 ng/mL, about 500 ng/mL, about 600 ng/mL, about 700 ng/mL, about 800 ng/mL, about 900 ng/mL, about 1,000 ng/mL. In some embodiments, the IFN $\gamma$  is present at a concentration of



about 100 ng/mL. In some embodiments, the IFN $\gamma$  is present at a concentration of about 200 ng/mL. In some embodiments, the IFN $\gamma$  is present at a concentration of about 400 ng/mL.

**[0043]** In some embodiments, the priming step comprises a combination of two or more of IL-2, IL-15 and IL-21. 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.

**[0044]** In some embodiments, the priming step comprises IL-2. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower. In some embodiments, the priming step comprises no added IL-2. In some embodiments, the priming step comprises IL-15 and/or IL-21 at a concentration of about 1 ng/mL to about 100 ng/mL. In some embodiments, the priming step comprises IL-15 and/or IL-21 at a concentration of about 10 ng/mL. In some embodiments, the priming step comprises IL-2 and IL-21. In some embodiments, the priming step comprises IL-2 at 3000 IU/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the priming step comprises IL-15 and IL-21. In some embodiments, the priming step comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.

**[0045]** The priming step may last for about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 18 hours, about 24 hours, about 30 hours, about 36 hours, about 48 hours. In some embodiments, the priming step lasts for 12 hours. In some embodiments, the priming step lasts for 24 hours. In some embodiments, the priming step lasts for 30 hours. In some embodiments, the priming step lasts for 36 hours. In some embodiments, the priming step lasts for 48 hours.

#### **D. Enriching Tumor Reactive TILs**

**[0046]** The present invention encompasses methods for the enrichment and expansion of a population of tumor-reactive T cells having a CD137<sup>+</sup> phenotype. In some embodiments, the population of TILs after the priming step is enriched for CD137<sup>+</sup> TILs (see Fig. 48). By way of example, a cell that is “CD137<sup>+</sup>” or that “expresses CD137” is contrasted herein to a cell that is CD137<sup>−</sup> or does not express a detectable level of CD137. A cell that is “CD137<sup>dim</sup>,” as used herein has a lower detectable level of CD137 expression than a CD137<sup>+</sup> cell or a “CD137<sup>bright</sup>” cell. In one embodiment, the tumor-reactive T cells are positive for both

CD137 and PD-1. By way of example, a cell that is “CD137+PD-1+” or that “expresses CD137 and PD-1” is contrasted herein to a cell that is CD137-PD-1+, CD137+PD-1– or does not express a detectable level of either CD137 or PD-1.

[0047] The present invention encompasses a method for isolating CD137+ cells using a straightforward direct antibody-based purification system for isolating such cells. However, any cellular isolation methodology can be used to isolate the cells of the invention from a biological sample. For example, an antibody that binds to CD137 can be bound to a physical support, such as a magnetic bead, a Dynal bead, a microbead, a column, an adsorption column, and an adsorption membrane. Conjugating an antibody to a physical support is well known in the art. Alternatively, an antibody conjugated to a physical support, such as a magnetic bead, can be purchased from a variety of sources, such as Milteny Biotec (Auburn, Calif.).

[0048] Literature shows that some tumor-reactive TILs reside in the CD137+ population. CD39 is more selective and is expressed by the majority of the putative CD4 tumor reactive (PD1+ICOS+) population. CD200 is expressed by the majority of the putative tumor reactive CD4+CXCL13 population. Therefore, in some embodiments, the enrichment step may further comprise selecting TILs that express CD200 and/or CD39. Further, OX40 is a known activation marker for CD4 T cells that would potentially be upregulated 24 hours after TCR stimulation in digest culture. Therefore, in some embodiments, the enrichment step may further comprise selecting TILs that express CD200, CD39 and/or OX40.

[0049] By including an anti-CD200 antibody, an anti-CD39 antibody and/or an anti-OX40 antibody in addition to an anti-CD137 antibody, the yield of tumor reactive TILs will be increased by the method disclosed herein, which still removing a very significant fraction of the bystander TILs. In some embodiments, the tumor reactive TILs may be a population of CD200+ cells. In some embodiments, the tumor reactive TILs may be a population of CD39+ cells. In some embodiments, the tumor reactive TILs may be a population of OX40+ cells.

[0050] In some embodiments, the enriching the population of TILs for CD137+ TILs comprises contacting the population of TILs with anti-CD137 antibody immobilized on a bead. In some embodiments, the method further comprises contacting the population of TILs with an anti-CD39 antibody immobilized on a bead. In some embodiments, the method further comprises contacting the population of TILs with an anti-CD200 antibody immobilized on a

bead. In some embodiments, the method further comprises contacting the population of TILs with an anti-OX40 antibody immobilized on a bead.

[0051] A variety of antibodies are useful in the present invention. As will be understood by one skilled in the art, any antibody that can recognize and bind to an antigen of interest, such as CD137, CD200, CD39, or OX40, is useful in the present invention. Methods of making and using such antibodies are well known in the art. For example, polyclonal antibodies useful in the present invention are generated by immunizing rabbits according to standard immunological techniques well-known in the art.

#### **E. First Expansion**

[0052] In some embodiments, the tumor reactive TILs resulting from the enrichment step are cultured in a second cell culture medium comprising feeder cells. In some embodiments, the feeder cells are T cell-depleted feeder cells, such as T cell-depleted PBMCs (see Fig. 48).

[0053] In some embodiments, the tumor reactive TILs resulting from the enrichment step are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor reactive TILs resulting from the enrichment step are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. In some embodiments, the tumor reactive TILs are cultured for a period of days, generally from 3 to 14 days. In some embodiments, the tumor reactive TILs are cultured for a period of 5 to 11 days. In some embodiments, the tumor reactive TILs are cultured for a period of 7 to 10 days. In some embodiments, the tumor reactive TILs are cultured for a period of about 9 days.

[0054] In embodiments where TIL cultures are initiated in 24-well plates, for example, using Costar 24-well cell culture cluster, flat bottom (Corning Incorporated, Corning, NY, each well can be seeded with  $1 \times 10^6$  tumor digest cells or one tumor fragment in 2 mL of complete medium (CM) with IL-2 (6000 IU/mL; Chiron Corp., Emeryville, CA).

[0055] In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, CM for the first expansion consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10 cm<sup>2</sup> gas-permeable silicon bottom (for example, G-Rex10; Wilson Wolf Manufacturing, New Brighton, MN, each flask may be loaded with 10–40  $\times 10^6$  viable tumor digest cells or 5–30 tumor fragments in 10–40 mL of CM with IL-2. Both the G-

Rex10 and 24-well plates may be incubated in a humidified incubator at 37°C in 5% CO<sub>2</sub> and 5 days after culture initiation, half the media may be removed and replaced with fresh CM and IL-2 and after day 5, half the media may be changed every 2–3 days.

[0056] 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.

[0057] 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.

[0058] 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<sup>+</sup>, Al<sup>3+</sup>, Ba<sup>2+</sup>, Cd<sup>2+</sup>, Co<sup>2+</sup>, Cr<sup>3+</sup>, Ge<sup>4+</sup>, Se<sup>4+</sup>, Br, T, Mn<sup>2+</sup>, P, Si<sup>4+</sup>, V<sup>5+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, Rb<sup>+</sup>, Sn<sup>2+</sup> and Zr<sup>4+</sup>. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[0059] In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[0060] 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.

[0061] In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55μM.

[0062] In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell

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

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

[0063] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

[0064] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55μM.

[0065] 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ ,  $\text{Br}$ ,  $\text{T}$ ,  $\text{Mn}^{2+}$ ,  $\text{P}$ ,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[0066] 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.

[0067] 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 11 below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading “A Preferred Embodiment of the 1X Medium” in Table 11. 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 11 below.

TABLE 11: 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 <sub>4</sub> (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 <sup>®</sup> I                    | 83,000                                              | 5000-50,000                                     | 12,500                                             |

**[0068]** 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  $\mu$ M), 2-mercaptoethanol (final concentration of about 100  $\mu$ M).

**[0069]** 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<sup>™</sup> OpTmizer<sup>™</sup> was used as the basal cell medium, and supplemented with either 0, 2%, 5%, or 10% CTS<sup>™</sup> Immune Cell Serum Replacement.

[0070] 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  $\beta$ ME; also known as 2-mercaptoethanol, CAS 60-24-2).

[0071] After preparation of the tumor fragments, the resulting cells (*i.e.*, fragments) are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum (or, in some cases, as outlined herein, in the presence of aAPC cell population) with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 10 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, the growth media during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the IL is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of  $20\text{-}30 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of  $20 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of  $25 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of  $30 \times 10^6$  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  $6 \times 10^6$  IU/mg of IL-2. In some embodiments, the first expansion culture media comprises about 10,000 IU/mL of IL-2, about 9,000 IU/mL of IL-2, about 8,000 IU/mL of IL-2, about 7,000 IU/mL of IL-2, about 6000 IU/mL of IL-2 or about 5,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 9,000 IU/mL of IL-2 to about 5,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 8,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 7,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 6,000 IU/mL of IL-2. In 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 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, between 1000 and 5000 IU/mL, or about 8000 IU/mL of IL-2.

[0072] In some embodiments, the first expansion can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the first expansion can proceed for 1 day to 14 days. In some embodiments, the first expansion can proceed for 2 days to 14 days. In some embodiments, the first expansion can proceed for 3 days to 14 days. In some embodiments, the first expansion can proceed for 4 days to 14 days. In some embodiments, the first expansion can proceed for 5 days to 14 days. In some embodiments, the first expansion can proceed for 6 days to 14 days. In some embodiments, the first expansion can proceed for 7 days to 14 days. In some embodiments, the first expansion can proceed for 8 days to 14 days. In some embodiments, the first expansion can proceed for 9 days to 14 days. In some embodiments, the first expansion can proceed for 10 days to 14 days. In some embodiments, the first expansion can proceed for 11 days to 14 days. In some embodiments, the first expansion can proceed for 12 days to 14 days. In some embodiments, the first expansion can proceed for 13 days to 14 days. In some embodiments, the first expansion can proceed for 14 days. In some embodiments, the first expansion can proceed for 1 day to 11 days. In some embodiments, the first expansion can proceed for 2 days to 11 days. In some embodiments, the first expansion can proceed for 3 days to 11 days. In some embodiments, the first expansion can proceed for 4 days to 11 days. In some embodiments, the first expansion can proceed for 5 days to 11 days. In some embodiments, the first expansion can proceed for 6 days to 11 days. In some embodiments, the first expansion can proceed for 7 days to 11 days. In some embodiments, the first expansion can proceed for 8 days to 11 days. In some embodiments, the first expansion can proceed for 9 days to 11 days. In some embodiments, the first expansion can proceed for 10 days to 11 days. In some embodiments, the first expansion can proceed for 11 days. In some embodiments, the first expansion can proceed for 5 days to 7 days. In some embodiments, the first expansion can proceed for 6 days to 7 days. In some embodiments, the

first expansion can proceed for 7 days to 12 days. In some embodiments, the first expansion can proceed for 8 days to 12 days. In some embodiments, the first expansion can proceed for 9 days to 12 days. In some embodiments, the first expansion can proceed for 10 days to 12 days. In some embodiments, the first expansion can proceed for 7 days. In some embodiments, the first expansion can proceed for 9 days.

[0073] 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 single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

[0074] In some embodiments, the first cell culture medium comprises 6000 IU/mL of IL-2. In some embodiments, the first cell culture medium comprises 3000 IU/mL of IL-2. In some embodiments, the first cell culture medium comprises 2000 IU/mL of IL-2. In some embodiments, the first cell culture medium comprises 1000 IU/mL of IL-2.

#### **F. Activation**

[0075] In some embodiments, after the first expansion (pre-REP) step the TILs are activated by adding anti-CD3 agonist and anti-CD28 agonist, such as TransAct, to the culture medium and culturing for about 1 to 3 days, wherein the TILs will be transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs.

[0076] In some embodiments, the step of activating the second population of TILs (obtained from the first expansion or pre-REP step) can be performed for a period that is, is about, is less than, is more than, 1 day, 2 days, 3 days, or a range that is between any of the above values. For example, in some embodiments, the step of activating the second population of TILs is performed for about 1 day. In some embodiments, the step of activating the second population of TILs is performed for about 2 days. In some embodiments, the step of activating the second population of TILs is performed for about 3 days.

[0077] In some embodiments, the step of activating the second population of TILs (obtained from the first expansion or pre-REP step) is performed using anti-CD3 agonist and anti-CD28 agonist, such as TransAct. In some embodiments, the step of activating the second population of TILs is performed using TransAct at 1:10 dilution, at 1:17.5 dilution, at 1:20

dilution, at 1:25 dilution, at 1:30 dilution, at 1:40 dilution, at 1:50 dilution, at 1:60 dilution, at 1:70 dilution, at 1:80 dilution, at 1:90 dilution, or at 1:100 dilution.

[0078] In some embodiments, the step of activating the second population of TILs (obtained from the first expansion or pre-REP step) can be performed by adding the anti-CD3 agonist and anti-CD28 agonist, such as TransAct, to the first cell culture medium. In some embodiments, the step of activating the second population of TILs can be performed by replacing the first cell culture medium with a cell culture medium comprising the anti-CD3 agonist and anti-CD28 agonist, such as TransAct.

[00347] In some embodiments, the method may comprise activating the population of TILs for 1 day, 2 days, 3 days, or 4 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with a cytokine selected from the group consisting of IL-2, IL-15, IL-21, IL-7, and a combination thereof.

[00348] In some embodiments, the activating step comprises contacting the population of TILs with 20ng/mL IL-15. In some embodiments, the activating step comprises contacting the population of TILs with 10 ng/mL IL-7. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100. In some embodiments, the activating step is conducted after the first expansion step and before the second expansion step.

#### **G. Lentiviral Particle Production**

[00349] In some embodiments, recombinant lentiviral particles are produced using a recombinant expression vector, one or more helper plasmids encoding viral gag, pol, env, and rev, and/or a packaging cell line disclosed herein.

[00350] The following general steps may be used. First, culturing a population of packaging cells in a cell culture medium. Once sufficient numbers of packaging cells are obtained, the desired nucleic acids, such as a transfer vector comprising a nucleic acid molecule encoding the cytokines disclosed herein, can be introduced into the packaging cells. Additional nucleic acids that may be introduced into the packaging cells include plasmids that promote packaging, e.g., plasmids encoding viral gag, pol, env, and rev. The packaging cells then begin to produce recombinant lentiviral particles.

[00351] In some embodiments, HEK 293T cells are resuspended in virus culture medium (10%FBS, DMEM (high glucose, Glutamine), sodium pyruvate (1% w/v), sodium bicarbonate (0.075%) or HEPES, no Pen/strep).

[00352] On the next day, a Gag/Pol helper plasmid, a Rev helper plasmid, a BaEVTR or VSV-G helper plasmid, and a transfer vector are mixed at 1 µg/µL concentration at a 1:1 molar ratio. Following mixing, 30 µL of *TransIT*<sup>®</sup>-Lenti reagent (Mirus Bio) is added and incubated at room temperature for 10 min in 1mL Opti-MEM<sup>™</sup> media per HEK 293T dish. HEK 293T cells are then incubated at 37°C, 5% CO<sub>2</sub> for 2 days.

[00353] Culture supernatant is removed from dishes and fresh media is added to each dish, centrifuged at 200 g for 5 min to remove cellular debris, and then filtered using a .45 µm filter. Viral supernatant is then concentrated via ultracentrifugation for 2 hrs at 60,000 g. Following ultracentrifugation, supernatant is removed and viral pellet is redissolved in Opti-MEM<sup>™</sup> media. The viral particle harvesting step may be repeated to optimize viral production.

## H. Transduction

[00354] In some embodiments, after the activation step, the TILs are transduced with the recombinant lentiviral particle disclosed herein.

[00355] In some embodiments, the transduction step is performed at a TIL concentration of about  $10^4$  –  $10^6$  cells/mL. In some embodiments, the transduction step is performed at a TIL concentration of about  $10^4$  cells/mL. In some embodiments, the transduction step is performed at a TIL concentration of about  $10^5$  cells/mL. In some embodiments, the transduction step is performed at a TIL concentration of about  $10^6$  cells/mL. In some embodiments, the transducing step is performed at a multiplicity of infection (MOI) of about 10 to about 40. In some embodiments, the transducing step is performed at an MOI of about 10. In some embodiments, the transducing step is performed at an MOI of about 20. In some embodiments, the transducing step is performed at an MOI of about 30. In some embodiments, the transducing step is performed at an MOI of about 40.

[00356] In some embodiments, the transducing step is performed in a non-tissue culture 48 well plate coated with Retronectin (diluted 1:100 in PBS) by adding 250uL per well. The 48 well plate is then wrapped in parafilm and then incubated at 4°C overnight.

[00357] The coating solution is removed from Retronectin-coated plate and replaced with 250uL 2% BSA Fraction V. The plated is incubate at RT for 30min for blocking. The following is added:

- a. 100uL of TIL suspension (1e5 cells)
- b. 300uL CM2 + IL-15 (20ng/mL) + IL-7 (40ng/mL) + Lentibooster 1:50
- c. Lentivirus with MOI in a range of 10-40
- d. Adjust with CM2 w/o gentamicin, total volume 600uL per well

[00358] The plate is then centrifuged at 2000xg, 32 °C for 45min.

[00359] In some embodiments, the transducing step is conducted in the presence of RetroNectin or Vectofusin-1. In some embodiments, the transducing step comprises centrifugation. In some embodiments, the transducing step is conducted in the presence of Lentibooster. In some embodiments, the transducing step is conducted in the presence of Lentibooster at a ratio of 1:50.

[00360] In some embodiments, the transducing step is conducted after the first expansion step and before the second expansion step.

[00361] In some embodiments, the method further comprises resting the population of TILs for 1 day, 2 days, 3 days, or 4 days after the transducing step.

## I. Second Expansion

[0079] In some embodiments, the TIL cell population is expanded in number after initial bulk processing, pre-REP expansion, and genetic modification, wherein the expanded TILs have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. This further expansion is referred to herein as the second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (REP). The second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 agonist antibody, in a gas-permeable container.

[0080] In some embodiments, the 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, wherein the expanded TILs have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, the second expansion can proceed for 7 days, 8 days, 9 days, 10

days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the second expansion can proceed for about 7 days to about 14 days. In some embodiments, the second expansion can proceed for about 7 days to about 12 days. In some embodiments, the second expansion can proceed for about 7 days to about 10 days. In some embodiments, the second expansion can proceed for about 7 days to about 9 days. In some embodiments, the second expansion can proceed for about 8 days to about 9 days. In some embodiments, the second expansion can proceed for about 9 days. In some embodiments, the second expansion can proceed for about 10 days. In some embodiments, the second expansion can proceed for about 11 days.

**[0081]** In some embodiments, the second expansion can be performed in a gas permeable container using the methods of the present disclosure (including for example, expansions referred to as REP). 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 agonist 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  $\mu$ 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.

**[0082]** 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.

**[0083]** 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, or 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 does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

**[0084]** 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 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 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.

**[0085]** In some embodiments, REP and/or the second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. Media replacement is done (generally 2/3 media replacement via respiration with fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[0086]** In some embodiments, the second expansion (which can include processes referred to as the REP process) is shortened to 7-14 days, as discussed in the examples and figures, wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, the second expansion is shortened to 9 days.

**[0087]** In some embodiments, REP and/or the second expansion may be performed using T-175 flasks and gas permeable bags as previously described (Tran, *et al.*, *J. Immunother.* **2008**, *31*, 742-51; Dudley, *et al.*, *J. Immunother.* **2003**, *26*, 332-42) or gas permeable cultureware (G-Rex flasks), wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, the second expansion (including expansions referred to as rapid expansions) is performed in T-175 flasks, and about  $1 \times 10^6$  TILs suspended in 150 mL of media may be added to each T-175 flask. The TILs may be cultured in a 1 to 1 mixture of CM and AIM-V medium, supplemented with 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3. The T-175 flasks may be incubated at 37° C in 5% CO<sub>2</sub>, wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. Half the media may be exchanged on day 5 using 50/50 medium with 3000 IU per mL of IL-2. In some embodiments, on day 7 cells from two T-175 flasks may be combined in a 3 L bag and 300 mL of AIM V with 5% human AB serum and 3000 IU per mL of IL-2 was added to the 300 mL of TIL suspension. The number of cells in each bag was counted every day or two and fresh media was added to keep the cell count between  $0.5$  and  $2.0 \times 10^6$  cells/mL.

**[0088]** 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),  $5 \times 10^6$  or  $10 \times 10^6$  TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3), wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. The G-Rex 100 flasks may be incubated at 37°C in 5% CO<sub>2</sub>. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and centrifuged at 1500 rpm ( $491 \times g$ ) for 10 minutes. The TIL

pellets may be re-suspended with 150 mL of fresh medium with 5% human AB serum, 3000 IU per mL of IL-2, and added back to the original G-Rex 100 flasks. When TIL are expanded serially in G-Rex 100 flasks, on day 7 the TIL in each G-Rex 100 may be suspended in the 300 mL of media present in each flask and the cell suspension may be divided into 3 100 mL aliquots that may be used to seed 3 G-Rex 100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU per mL of IL-2 may be added to each flask. The G-Rex 100 flasks may be incubated at 37° C in 5% CO<sub>2</sub> and after 4 days 150 mL of AIM-V with 3000 IU per mL of IL-2 may be added to each G-REX 100 flask. The cells may be harvested on day 14 of culture.

[0089] 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),  $5 \times 10^6$  or  $10 \times 10^6$  TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3). The G-REX-100 (or G-REX100M) flasks may be incubated at 37°C in 5% CO<sub>2</sub>. 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 TILs 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 (or G-REX500M). 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. In some embodiments, the process employed varying centrifugation speeds (400g, 300g, 200g for 5 minutes) and varying numbers of repetitions.

[0090] In some embodiments, the second expansion (including expansions referred to as REP) is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. In some embodiments, media replacement is done until the cells are transferred to an

alternative growth chamber, wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, 2/3 of the media is replaced by aspiration of spent media followed by infusion with fresh media. In some embodiments, alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[0091]** In some embodiments, the second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below.

**[0092]** 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.

**[0093]** 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.

**[0094]** 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ ,  $\text{Br}$ ,  $\text{T}$ ,  $\text{Mn}^{2+}$ ,  $\text{P}$ ,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[0095] In some embodiments, the CTS™ OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[0096] 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.

[0097] In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR)

(ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55 $\mu$ M.

[0098] In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific)

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

[0099] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

[00100] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55µM.

[00101] 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ , Br, T,  $\text{Mn}^{2+}$ , P,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00102]** 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.

**[00103]** 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 12 below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading “A Preferred Embodiment of the 1X Medium” in Table 12. 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 12 below.

TABLE 12: 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 <sub>4</sub> (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 <sup>®</sup> I                    | 83,000                                              | 5000-50,000                                     | 12,500                                             |

[00104] 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  $\mu$ M), 2-mercaptoethanol (final concentration of about 100  $\mu$ M).

**[00105]** 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.

**[00106]** 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  $\beta$ BME; also known as 2-mercaptoethanol, CAS 60-24-2).

**[00107]** In some embodiments, the 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 single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

**[00108]** In some embodiments, the steps of the method are completed within a period of about 22 days. In some embodiments, the steps of the method are completed within a period of about 8 days. In some embodiments, the steps of the method are completed within a period of about 9 days. In some embodiments, the steps of the method are completed within a period of about 10 days. In some embodiments, the steps of the method are completed within a period of about 11 days. In some embodiments, the steps of the method are completed within a period of about 12 days. In some embodiments, the steps of the method are completed within a period of about 13 days. In some embodiments, the steps of the method are completed within a period of about 14 days. In some embodiments, the steps of the method are completed within a period of about 15 days. In some embodiments, the steps of the method are completed within a period of about 16 days. In some embodiments, the steps of the method are completed within a period of about 17 days. In some embodiments, the steps of the method are completed within a period of about 18 days. In some embodiments, the steps of the method are completed within a period of about 19 days. In some embodiments, the steps of the method are completed within a period of about 20 days. In some embodiments, the steps of the method are completed within a period of about 21 days.

In some embodiments, the steps of the method are completed within a period of about 22 days. In some embodiments, the steps of the method are completed within a period of about 23 days. In some embodiments, the steps of the method are completed within a period of about 24 days. In some embodiments, the steps of the method are completed within a period of about 25 days. In some embodiments, the steps of the method are completed within a period of about 26 days. In some embodiments, the steps of the method are completed within a period of about 27 days. In some embodiments, the steps of the method are completed within a period of about 28 days. In some embodiments, the steps of the method are completed within a period of about 29 days. In some embodiments, the steps of the method are completed within a period of about 30 days. In some embodiments, the steps of the method are completed within a period of about 31 days.

**[00109]** In some embodiments, the antigen presenting cells (APCs) are PBMCs. According to some embodiments, the PBMCs are irradiated. According to some embodiments, the PBMCs are allogeneic. According to some embodiments, the PBMCs are irradiated and allogeneic. According to some embodiments, the antigen-presenting cells are artificial antigen-presenting cells.

**[00110]** In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 1500 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 2000 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 2500 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 3000 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 3500 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 4000 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 4500 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 5000 IU/mL and 6000 IU/mL in the cell culture medium in the first expansion. In some embodiments, the IL-2 is present at an initial concentration of between 5500 IU/mL and 6000

[illegible]

embodiments, the IL-2 is present at an initial concentration of between 1500 IU/mL and 2000 IU/mL in the cell culture medium in the first expansion.

[00111] In some embodiments, the second expansion step, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL and the OKT-3 antibody is present at an initial concentration of about 30 ng/mL.

[00112] In some embodiments, the first expansion is performed using a gas permeable container. In some embodiments, the second expansion is performed using a gas permeable container.

[00113] In some embodiments, the first cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof. In some embodiments, the second cell culture medium and/or third culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

#### 1. Feeder Cells and Antigen Presenting Cells

[00114] In some embodiments, the second expansion procedures described herein require an excess of feeder cells during REP TIL expansion and/or during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

[00115] 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.

[00116] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

[00117] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second

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

**[00118]** In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/mL OKT3 antibody and 1000-6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/mL OKT3 antibody and 2000-5000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/mL OKT3 antibody and 2000-4000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/mL OKT3 antibody and 2500-3500 IU/mL IL-2.

**[00119]** 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.

**[00120]** In some embodiments, the second expansion procedures described herein require a ratio of about  $2.5 \times 10^9$  feeder cells to about  $100 \times 10^6$  TIL. In other embodiments, the second expansion procedures described herein require a ratio of about  $2.5 \times 10^9$  feeder cells to about  $50 \times 10^6$  TIL. In yet other embodiments, the second expansion procedures described herein require about  $2.5 \times 10^9$  feeder cells to about  $25 \times 10^6$  TIL.

**[00121]** In some embodiments, the second expansion procedures described herein require an excess of feeder cells during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

[00122] In some embodiments, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

## 2. Cytokines and Other Additives

[00123] 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.

[00124] Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILs is 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.

[00362] In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-2. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower. In some embodiments, the first cell culture medium or the second cell culture medium contains no added IL-2. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 1 ng/mL to about 100 ng/mL. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 10 ng/mL. In some embodiments, the first cell culture medium comprises IL-2 and IL-21. In some embodiments, the first cell culture medium comprises IL-2 at 3000 IU/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and a protein kinase B (AKT) inhibitor. In some embodiments, the AKT inhibitor is selected from the group consisting of ipatasertib, GSK690693, GSK2141795, GSK2110183, AZD5363, GDC-0068, AT7867, CCT128930, MK-2206, BAY 1125976, Perifosine, Oridonin, Herbacetin, Tehranolide, Isoliquiritigenin, Scutellarin, and Honokiol.

[00363] In some embodiments, the first expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 7-11 days.

### 3. Modifiers of T Cell Metabolism

[00364] In vitro expansion alters TIL cellular state, which correlates with ACT efficacy (Chiffelle, J., et al., *bioRxiv* **2023**, the content of which is hereby incorporated by reference in its entirety). Rapidly expanding TILs have bioenergetic and biosynthetic needs. Increasing the availability of essential cofactors such as L-arginine and NAD<sup>+</sup> may support synthesis of major biomass components. To functionally reinvigorate cells during in vitro expansion may produce TILs with renewed effector features.

[00365] Therefore, provided herein are modifiers of T cell metabolism that increase availability of essential cofactors L-arginine and NAD<sup>+</sup> to support synthesis of major biomass components in TILs, which may be added to the second expansion (REP) of TILs.

[00366] L-arginine, a building block for protein synthesis, improves T cell function via metabolic modification (Geiger R., et al., *Cell* **2016**: 167; 829-842; Fultang, L., *Blood* **2020**; 136: 1155-60; the contents of which are hereby incorporated by reference in their entireties).

[00367] NAD<sup>+</sup> (nicotinamide adenine dinucleotide), an electron acceptor, is heavily consumed during biomass synthesis, and supplementation improves T cell function (Canto C, et al., *Cell Metabolism* **2015**; 22:31-53; Wang Y., et al., *Cell Reports* **2021**; 36:1-12; the contents of which are hereby incorporated by reference in their entireties).

[00368] NAD<sup>+</sup> (Nicotinamide adenine dinucleotide) is a central coenzyme in cellular metabolism, particularly in redox reactions where it cycles between oxidized (NAD<sup>+</sup>) and reduced (NADH) states. In the context of TILs, NAD<sup>+</sup> plays a critical role in modulating their metabolic fitness and function. TILs operate in the tumor microenvironment, which is often characterized by nutrient scarcity and hypoxia. In this challenging microenvironment, TILs must adapt their metabolism to sustain their anti-tumor activity.

[00369] NAD<sup>+</sup> enhances the metabolic fitness of T cells in several ways:

[00370] Glycolysis and the Pentose Phosphate Pathway (PPP): Although glycolysis does not directly use NAD<sup>+</sup>, the regeneration of NAD<sup>+</sup> from NADH is critical to maintain glycolytic flux, which is particularly important in hypoxic conditions where mitochondrial respiration is limited. The PPP, which branches from glycolysis, relies on NADP<sup>+</sup> (the phosphorylated



form of NAD<sup>+</sup>) for the generation of ribose-5-phosphate and for the reduction of glutathione, contributing to nucleotide synthesis and antioxidant defense mechanisms.

**[00371]** Mitochondrial Function: NAD<sup>+</sup> is essential in the mitochondria as it is a key electron acceptor in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Enhanced mitochondrial function can support the differentiation and survival of memory T cells, which are crucial for sustained anti-tumor immunity.

**[00372]** Sirtuins Activation: Sirtuins are a family of NAD<sup>+</sup>-dependent deacetylases that regulate cellular metabolism, stress responses, and inflammation. By activating sirtuins, NAD<sup>+</sup> can promote the formation of memory T cells and enhance their anti-tumor function.

**[00373]** PARP Activity: NAD<sup>+</sup> is a substrate for poly(ADP-ribose) polymerases (PARPs) which are involved in DNA repair and genomic stability. By facilitating the function of PARPs, NAD<sup>+</sup> helps maintain TIL integrity in the face of DNA-damaging conditions within tumors.

**[00374]** To replicate and enhance the benefits provided by NAD<sup>+</sup> to TILs, both pharmacological and genetic approaches can be considered:

**[00375]** Pharmacologic Approaches:

- NAD<sup>+</sup> Precursors: Boosting NAD<sup>+</sup> via administration of NAD<sup>+</sup> precursors such as nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN) can increase intracellular NAD<sup>+</sup> levels and thereby support TIL metabolism and function.
- NAD<sup>+</sup>: Boosting availability of NAD<sup>+</sup> metabolic substrate directly to TILs
- Sirtuin Activators: Compounds like resveratrol or SRT1720 can activate sirtuins, thus mimicking the effect of high NAD<sup>+</sup> levels and promoting TIL anti-tumor activity.
- PARP Inhibitors: While PARP activity is NAD<sup>+</sup> dependent and can be beneficial, excessive activation can deplete cellular NAD<sup>+</sup> reserves. PARP inhibitors can help preserve NAD<sup>+</sup> levels under stress conditions, potentially supporting T cell function.
- CD38 Inhibitors: CD38 uses NAD<sup>+</sup> to generate ADP-ribose and cyclic ADP-ribose, which are important for calcium signaling but also deplete NAD<sup>+</sup>. Inhibitors of CD38 may help preserve NAD<sup>+</sup> levels in TILs.

**[00376]** Genetic Approaches:

- Overexpression of NAMPT: This is the rate-limiting enzyme in the NAD<sup>+</sup> salvage pathway. Overexpressing NAMPT can boost the intracellular levels of NAD<sup>+</sup> in TILs.

- Manipulation of Sirtuin Genes: Genetic modification to overexpress sirtuin genes can potentially mimic the effect of high NAD<sup>+</sup> levels and enhance TIL function.
- Gene Silencing of NAD<sup>+</sup> Consuming Enzymes: Silencing or knocking down genes encoding for enzymes that heavily consume NAD<sup>+</sup>, like CD38 or PARP family members, might conserve NAD<sup>+</sup> for metabolic processes essential for TILs.
- TCA Cycle and OXPHOS Support: Enhancing genes involved in mitochondrial biogenesis and function can support the efficient use of NAD<sup>+</sup> and maintain TIL metabolic fitness.

[00377] Pharmacologic and genetic tools to augment NAD<sup>+</sup> utilization in TILs center on specific molecular targets and pathways. In some embodiments, exemplary tools for augment NAD<sup>+</sup> utilization focus on one or more of the following functions: mitochondrial biogenesis, promoting effective energy utilization, and fostering an anti-tumor phenotype. In embodiments, such tools are implemented in a concerted manner, with attention to the balance between enhancing TIL function and avoiding over-stimulation that could lead to exhaustion or apoptosis. In some embodiments, the heterogeneity within the TIL population and the unique microenvironment of each tumor when designing these interventions are taken into consideration. The modulation of mitochondrial function through pharmacological and genetic approaches could thus be a powerful strategy to enhance the efficacy of adoptive TIL therapies. Tools for augmenting NAD<sup>+</sup> utilization are further detailed below.

#### **Pharmacological Manipulation of NAD<sup>+</sup> Pathways:**

##### **1. NAD<sup>+</sup> Precursors and NAD<sup>+</sup>:**

- o **Nicotinamide Riboside (NR)** and **Nicotinamide Mononucleotide (NMN)** are NAD<sup>+</sup> precursors. They are converted to NAD<sup>+</sup> via the salvage pathway involving the enzyme nicotinamide riboside kinase (NRK1/2) for NR and NMN adenylyltransferase (NMNAT1/2/3) for NMN.
- o **Nicotinic Acid (NA)** can also be used, which is converted into NAD<sup>+</sup> via the Preiss-Handler pathway, although it can cause flushing due to prostaglandin-mediated vasodilation.
- o **Nicotinamide dinucleotide (NAD<sup>+</sup>)** itself can also be used, as it has been shown to cross the lipid bilayer and enter the mitochondria.

##### **2. Sirtuin Modulators:**

- **Resveratrol** is a sirtuin-activating compound (STAC) that enhances SIRT1 activity, which in turn can deacetylate and activate peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), a master regulator of mitochondrial biogenesis.
  - **SRT1720**, another STAC, has been shown to selectively activate SIRT1, leading to enhanced mitochondrial function and possible improvement in TIL survival and function.
3. **PARP Inhibitors:**
- Compounds like **Olaparib** and **Niraparib** can inhibit PARP enzymes, thus conserving NAD<sup>+</sup> for other metabolic processes critical for T cell function and potentially reducing the metabolic competition between PARPs and SIRT1 for NAD<sup>+</sup>.
4. **CD38 Inhibitors:**
- **Isatuximab** and **Daratumumab** are monoclonal antibodies that target CD38, which is responsible for NAD<sup>+</sup> degradation. Small molecule inhibitors of CD38 are also in development and could be repurposed to enhance NAD<sup>+</sup> levels in TILs.
5. **Mitochondrial Metabolic Modulators:**
- **Metformin** has been shown to activate AMP-activated protein kinase (AMPK), which in turn activates PGC-1 $\alpha$ , improving mitochondrial biogenesis and potentially restoring TIL function.
  - **Pioglitazone**, a PPAR $\gamma$  agonist, might also enhance mitochondrial biogenesis and function in TILs through PGC-1 $\alpha$  induction.
  - **Bezafibrate**: A pan-PPAR agonist that can induce PGC-1 $\alpha$ , enhancing mitochondrial function and fatty acid oxidation

#### **Mitochondrial Biogenesis and Dynamics:**

1. **PGC-1 $\alpha$  Activation:**
  - Specific activators of PGC-1 $\alpha$  or direct overexpression via gene delivery systems could be used to stimulate mitochondrial biogenesis.
2. **Mitochondrial Dynamics:**
  - Manipulating genes involved in mitochondrial fusion (e.g., **MFN1/2**, **OPA1**) and fission (e.g., **DRP1**, **FIS1**) to maintain mitochondrial network integrity and function.

**Genetic Tools:****1. Overexpression of NAD<sup>+</sup> Synthesis Genes:**

- *NAMPT*: Overexpression can increase the salvage pathway flux, boosting NAD<sup>+</sup> levels.
- *NMNAT1/2/3*: Increased expression of NMN adenylyltransferases to catalyze the conversion of NMN to NAD<sup>+</sup>.

**2. Sirtuin Gene Manipulation:**

- *SIRT1-7*: Overexpressing Sirtuin genes, with a focus on SIRT1 for its role in mitochondrial biogenesis, SIRT3 for mitochondrial protein deacetylation, and SIRT6 for maintaining genomic stability.

**3. Upregulation of TCA Cycle and OXPHOS Genes:**

- *PC (Pyruvate Carboxylase)*: Enhancing anaplerotic reactions to replenish TCA cycle intermediates.
- *PDK (Pyruvate Dehydrogenase Kinase) Inhibitors*: Genetic silencing of PDK to increase PDH activity and promote glucose oxidation.

**4. Downregulation of NAD<sup>+</sup> Consuming Enzymes:**

- Using CRISPR/Cas9 or siRNA approaches to knockdown CD38 or PARP family members to conserve NAD<sup>+</sup> for enhancing TIL function.

**5. Promotion of Mitochondrial DNA Stability and Biogenesis:**

- *TFAM (Mitochondrial Transcription Factor A)*: Overexpression can promote mitochondrial DNA replication and transcription, thus enhancing mitochondrial function.
- *OGG1 or MUTYH*: Overexpression to enhance base-excision repair and maintain mitochondrial DNA integrity.

**6. Redox Balance:**

- *GCLC/GCLM (Glutamate-Cysteine Ligase Catalytic and Modifier Subunit)*: Overexpression to increase glutathione synthesis, a key antioxidant within mitochondria.

**7. Metabolic Reprogramming:**

- *CPT1A (Carnitine Palmitoyltransferase 1A)*: Genetic modification to boost fatty acid oxidation, which could sustain TIL function under nutrient-deprived conditions.

**8. Mitophagy and Autophagy Regulators:**

- *PRKN* (*Parkin*) and *PINK1*: Enhance mitophagy to remove dysfunctional mitochondria.
- *ATG5* or *ATG7*: Modulation of autophagy-related genes to balance energy and organelle quality control in TILs.

**[00378]** In some embodiments, these strategies may be optimized for dose, timing, and delivery mechanisms to ensure the TILs are metabolically enhanced without inducing detrimental effects such as apoptosis or senescence. In embodiments, such strategies are also assessed to avoid the promotion of tumor cell survival or immune evasion. In some embodiments, a combination of these strategies, informed by the specific metabolic deficiencies observed in the TILs isolated from individual tumors, provides a potent and personalized enhancement of TIL function for adoptive cell therapies.

| Method/Compound                   | Mechanism of Action                                                                   | Target Process/Pathway                | Potential Use in TILs                                     | Considerations/Challenges                          |
|-----------------------------------|---------------------------------------------------------------------------------------|---------------------------------------|-----------------------------------------------------------|----------------------------------------------------|
| Nicotinamide Riboside (NR)        | Precursor to NAD <sup>+</sup> ; converted to NMN and then to NAD <sup>+</sup>         | NAD <sup>+</sup> salvage pathway      | Enhance TIL metabolism and function                       | Dosage optimization to avoid side effects          |
| Nicotinamide Mononucleotide (NMN) | Direct precursor to NAD <sup>+</sup> ; converted to NAD <sup>+</sup> by NMNAT enzymes | NAD <sup>+</sup> salvage pathway      | Improve TIL anti-tumor activity                           | Stability and cellular uptake considerations       |
| Niacin (Vitamin B3)               | Converted to NAD <sup>+</sup> via the Preiss-Handler pathway                          | NAD <sup>+</sup> biosynthesis pathway | Support TIL energy generation and DNA repair              | Flushing reaction at high doses                    |
| NAMPT Inhibitors                  | Increase NAD <sup>+</sup> levels by inhibiting its degradation                        | NAD <sup>+</sup> salvage pathway      | Potentially extend TIL lifespan                           | Risk of systemic NAD <sup>+</sup> depletion        |
| PARP Inhibitors                   | Prevent NAD <sup>+</sup> depletion by inhibiting PARP enzymes                         | DNA repair pathway                    | Preserve NAD <sup>+</sup> for metabolic functions in TILs | Balance between inhibiting PARP and cancer therapy |

| Method/Compound            | Mechanism of Action                                                    | Target Process/Pathway                                  | Potential Use in TILs                               | Considerations/Challenges                          |
|----------------------------|------------------------------------------------------------------------|---------------------------------------------------------|-----------------------------------------------------|----------------------------------------------------|
| Sirtuin Activators         | Increase NAD <sup>+</sup> utilization efficiency                       | Sirtuin-mediated deacetylation                          | Enhance TIL mitochondrial function                  | Long-term effects on TILs are not fully understood |
| CD38 Inhibitors            | Reduce NAD <sup>+</sup> degradation                                    | ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase pathway | Increase NAD <sup>+</sup> bioavailability for TILs  | May affect calcium signaling                       |
| Ketogenic Diet             | May elevate NAD <sup>+</sup> levels via increased fatty acid oxidation | Metabolic shift to ketone body usage                    | Could indirectly support TILs in vivo               | Feasibility in patients and impact on immune cells |
| Caloric Restriction        | Can upregulate NAD <sup>+</sup> biosynthesis pathways                  | General metabolic downregulation                        | Potentially increase TIL efficacy                   | Patient compliance and nutritional concerns        |
| Exercise                   | Upregulates NAD <sup>+</sup> biosynthesis and salvage pathways         | Enhanced muscle contraction and energy demand           | Indirect systemic effects on immune function        | Direct impact on TILs is unclear                   |
| NAD <sup>+</sup> Infusions | Direct supplementation of NAD <sup>+</sup>                             | Bypasses NAD <sup>+</sup> biosynthesis pathways         | Immediate replenishment of NAD <sup>+</sup> in TILs | Risk of imbalances and homeostasis disruption      |

| Method/Compound | Mechanism of Action                         | Target Process/Pathway                  | Potential Use in TILs                 | Considerations/Challenges                      |
|-----------------|---------------------------------------------|-----------------------------------------|---------------------------------------|------------------------------------------------|
| Gene Therapy    | Overexpression of NAD+ biosynthesis enzymes | Targeted enhancement of NAD+ production | Sustained increase in TIL NAD+ levels | Vector design, delivery, and integration risks |

[00379] The evolutionary conserved Wnt/ $\beta$ -catenin pathway is critically involved in pluripotency and differentiation of embryonic stem cells via maintenance of DNA methylation patterns promoting epigenetic stability necessary for correct cell fate decisions during states of high cell division (Clevers, H. and R. Nusse, *Cell*, **2012**. 149(6): p. 1192-205). In humans, rapid recall of memory T cells upon re-exposure to antigen suggests the requirement of a stable epigenetic program. Most TIL-based ACT protocols for solid tumors are generated from terminally differentiated effector memory T cells resulting from repetitive rounds of TCR ligation during the ex vivo phase (Hinrichs, C.S. and S.A. Rosenberg, *Immunol Rev*, **2014**. 257(1): p. 56-71). However, the critical involvement of DNA methylation in shaping expansion, memory state status and thus functionality of antigen-specific TILs undergoing TCR ligation-dependent expansion ex vivo is not known (Abdelsamed, H.A., et al., *J Exp Med*, **2017**. 214(6): p. 1593-1606).

[00380] There is evidence to show that pharmacological activation of the Wnt/ $\beta$ -catenin signaling reprograms tumor-specific CD8+ TILs in tumor-bearing mice by increasing expression of Tcf1 (encoded by Tcf7) (Gattinoni, L., et al., *Nat Med*, **2009**. 15(7): p. 808-13). In human melanoma, CD8+TCF7+ uncovers a subset of TILs with a memory precursor-like cell state with bystander cytotoxic function and is associated with positive outcome to immune checkpoint blockade (Li, H., et al., *Cell*, **2019**. 176(4): p. 775-789 e18; Sade-Feldman, M., et al., *Cell*, **2018**. 175(4): p. 998-1013 e20). Downregulation of the components of the Wnt pathway has been reported in PD1+ CD8+ memory TILs in vivo and a further downregulation upon *in vitro expansion* in all T cell populations (Lipp, J.J., et al., *Oncoimmunology*, **2022**. 11(1): p. 2019466), whereas compensating the lack of Wnt signaling through pharmacological inhibition of GSK3 $\beta$  improves the effector function of in vitro expanded populations. As Wnt signaling is known to arrest the development of effector



CD8<sup>+</sup> T cells, it also might be involved in epigenetically reprogramming TILs. GSK-3 is a positive regulator of PD-1 expression in CD8<sup>+</sup> T cells and GSK-3 inhibition enhances T cell function, and is effective in controlling tumor growth (Taylor, A., et al., *Immunity*, **2016**, 44(2): p. 274-86; Steele, L., et al., *iScience*, **2021**, 24(6): p. 102555). Besides being a central regulator of PD-1, GSK-3 also negatively regulates Lymphocyte Activation Gene-3 (LAG-3) expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells and small molecule inhibitors of GSK-3 were more effective than LAG-3 blockade alone in suppressing tumor growth in murine model of melanoma (Rudd, C.E., et al., *Cell Rep*, **2020**, 30(7): p. 2075-2082 e4). More recently, it was demonstrated that Wnt activation promotes human memory T cell polyfunctionality via epigenetic regulator PRMT1 (Sung, B.Y., et al., *J Clin Invest*, **2022**, 132(2)).

[00381] Therefore, in some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.

#### J. Harvest TILs

[00125] After the second expansion step, cells can be harvested. TILs can be harvested in any appropriate and sterile manner, including for example by centrifugation. Methods for TIL harvesting are well known in the art and any such know methods can be employed with the present process. In some embodiments, TILs are harvested using an automated system.

[00126] Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing systems is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term "LOVO cell processing system" also refers to any instrument or device manufactured by any vendor that can pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

[00127] In some embodiments, the harvest is performed from a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

[00128] In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in the Examples is employed.

#### **K. Final Formulation and Transfer to Infusion Container**

[00759] After the steps as outlined in detailed above and herein are complete, 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, such as an infusion bag, for use in administration to a patient. 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.

[00760] 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.

[00129] 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.

[00130] In some embodiments, TILs 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 by methods described in 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.

#### **L. Closed Systems for TIL Manufacturing**

[00131] The present invention provides for the use of closed systems during the TIL culturing process. Such closed systems allow for preventing and/or reducing microbial contamination, allow for the use of fewer flasks, and allow for cost reductions. In some embodiments, the closed system uses two containers.

[00132] Such closed systems are well-known in the art and can be found, for example, at <http://www.fda.gov/cber/guidelines.htm> and <https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076779.htm>.

[00133] Sterile connecting devices (STCDs) produce sterile welds between two pieces of compatible tubing. This procedure permits sterile connection of a variety of containers and tube diameters. In some embodiments, the closed systems include luer lock and heat-sealed systems as described in the Examples. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in the examples is employed. In some embodiments, the TILs are formulated into a final product formulation container according to the methods described herein in the examples.

[00134] In some embodiments, the closed system uses one container from the time the tumor fragments are obtained until the TILs are ready for administration to the patient or cryopreserving. In some embodiments when two containers are used, the first container is a closed G-container (such as G-rex100M series or G-rex500M series flasks) and the population of TILs is centrifuged and transferred to an infusion bag without opening the first

closed G-container. In some embodiments, when two containers are used, the infusion bag is a HypoThermosol-containing infusion bag. A closed system or closed TIL cell culture system is characterized in that once the tumor sample and/or tumor fragments have been added, the system is tightly sealed from the outside to form a closed environment free from the invasion of bacteria, fungi, and/or any other microbial contamination.

**[00135]** In some embodiments, the reduction in microbial contamination is between about 5% and about 100%. In some embodiments, the reduction in microbial contamination is between about 5% and about 95%. In some embodiments, the reduction in microbial contamination is between about 5% and about 90%. In some embodiments, the reduction in microbial contamination is between about 10% and about 90%. In some embodiments, the reduction in microbial contamination is between about 15% and about 85%. In some embodiments, the reduction in microbial contamination is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or about 100%.

**[00136]** The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

**[00137]** Moreover, pH, carbon dioxide partial pressure and oxygen partial pressure of the TIL cell culture environment each vary as the cells are cultured. Consequently, even though a medium appropriate for cell culture is circulated, the closed environment still needs to be constantly maintained as an optimal environment for TIL proliferation. To this end, it is desirable that the physical factors of pH, carbon dioxide partial pressure and oxygen partial pressure within the culture liquid of the closed environment be monitored by means of a sensor, the signal whereof is used to control a gas exchanger installed at the inlet of the culture environment, and the that gas partial pressure of the closed environment be adjusted in real time according to changes in the culture liquid so as to optimize the cell culture environment. In some embodiments, the present invention provides a closed cell culture system which incorporates at the inlet to the closed environment a gas exchanger equipped with a monitoring device which measures the pH, carbon dioxide partial pressure and oxygen partial pressure of the closed environment, and optimizes the cell culture environment by automatically adjusting gas concentrations based on signals from the monitoring device.

[00138] In some embodiments, the pressure within the closed environment is continuously or intermittently controlled. That is, the pressure in the closed environment can be varied by means of a pressure maintenance device for example, thus ensuring that the space is suitable for growth of TILs in a positive pressure state, or promoting exudation of fluid in a negative pressure state and thus promoting cell proliferation. By applying negative pressure intermittently, moreover, it is possible to uniformly and efficiently replace the circulating liquid in the closed environment by means of a temporary shrinkage in the volume of the closed environment.

[00139] In some embodiments, additional equipment, such as an electroporator (e.g., a Neon electroporator) is a component of an all-closed system. In some embodiments, optimal culture components for proliferation of the TILs can be substituted or added, and including factors such as IL-2 and/or OKT3, as well as combination, can be added.

[00140] In other embodiments, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-10. In other embodiments, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-100M. In other embodiments, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-500M.

## **VII. Method of Making a Population of TILs**

[00382] Methods of making a population of TILs (e.g., the Gen 2 process) has been described in International Patent Application Publication No. WO 2018/182817 A1 and International Patent Application Publication No. WO 2019/190579 A1, the disclosures of which are incorporated by reference herein in their entireties.

[00383] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising culturing the population of TILs in a cell culture medium, wherein the cell culture medium comprises IL-15, IL-21, and L-Arginine.

[00384] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising culturing the population of TILs in a cell culture medium, wherein the cell culture medium comprises IL-15, IL-21, L-Arginine, and NAD<sup>+</sup>.

[00385] Some embodiments disclosed herein provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising:

- a) performing a first expansion by culturing a population of TILs in a first cell culture medium; and
- b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, and L-arginine.

[00386] Some embodiments disclosed herein provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising:

- a) performing a first expansion by culturing a population of TILs in a first cell culture medium; and
- b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, L-arginine, and NAD<sup>+</sup>.

[00387] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium for about 3-14 days; b) performing a second expansion by culturing the population of TILs in a second cell culture medium for about 7-14 days, wherein the second cell culture medium comprises IL-15, IL-21, and L-Arginine.

[00388] Some embodiments of the present disclosure provide a method of making a population of tumor-infiltrating lymphocytes (TILs), comprising: a) performing a first expansion by culturing a population of TILs in a first cell culture medium for about 3-14 days; b) performing a second expansion by culturing the population of TILs in a second cell culture medium for about 7-14 days, wherein the second cell culture medium comprises IL-15, IL-21, L-Arginine, and NAD<sup>+</sup>.

[00389] Some embodiments of the present disclosure provide a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

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

- (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs, and wherein the transition from step (a) to step (b) occurs without opening the system;
- (c) performing a second expansion by culturing the second population of TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;
- (d) harvesting the therapeutic population of TILs obtained from step (c), wherein the transition from step (c) to step (d) occurs without opening the system, and wherein the therapeutic population of TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
- (e) transferring the harvested TIL population from step (d) to an infusion bag, wherein the transfer from step (d) to (e) occurs without opening the system, and
- (f) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

**[00390]** Some embodiments of the present disclosure provide a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

- (a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;
- (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs, and wherein the transition from step (a) to step (b) occurs without opening the system;

- (c) performing a second expansion by culturing the second population of TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;
- (d) harvesting the therapeutic population of TILs obtained from step (c), wherein the transition from step (c) to step (d) occurs without opening the system, and wherein the therapeutic population of TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
- (e) transferring the harvested TIL population from step (d) to an infusion bag, wherein the transfer from step (d) to (e) occurs without opening the system, and
- (f) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

**A. Obtain Patient Tumor Sample**

[00141] In general, TILs are initially obtained from a patient tumor sample (“primary TILs”) and then expanded into a larger population for further manipulation as described herein.

[00142] A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In some embodiments, multilesional sampling is used. In some embodiments, surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells includes multilesional sampling (*i.e.*, obtaining samples from one or more tumor sites and/or locations in the patient, as well as one or more tumors in the same location or in close proximity). In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of 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 endometrial cancer, cervical cancer, head and neck cancer (including, for example, head and neck squamous cell carcinoma (HNSCC)), glioblastoma (GBM), gastrointestinal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, and non-small cell lung carcinoma. In some embodiments, useful TILs are obtained from a melanoma.

**[00143]** Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 mm<sup>3</sup>, with from about 2-3 mm<sup>3</sup> being particularly useful. In some embodiments, the TILs are cultured from these fragments using enzymatic tumor digests. Such tumor digests may be produced by incubation in enzymatic media (*e.g.*, Roswell Park Memorial Institute (RPMI) 1640 buffer, 2 mM glutamate, 10 mcg/mL gentamicine, 30 units/mL of dNase and 1.0 mg/mL of collagenase) followed by mechanical dissociation (*e.g.*, using a tissue dissociator). Tumor digests may be produced by placing the tumor in enzymatic media and mechanically dissociating the tumor for approximately 1 minute, followed by incubation for 30 minutes at 37 °C in 5% CO<sub>2</sub>, 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.

#### **B. Tumor Fragmentation and/or Digest**

**[00144]** As indicated above, in some embodiments, the TILs are derived from solid tumors. In some embodiments, the solid tumors are not fragmented. In some embodiments, the solid tumors are not fragmented and are subjected to enzymatic digestion as whole tumors. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease for 1-2 hours. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, dNase, and neutral protease for 1-2 hours at 37°C, 5% CO<sub>2</sub>. In some embodiments, the tumors

are digested in in an enzyme mixture comprising collagenase, dNase, and neutral protease for 1-2 hours at 37°C, 5% CO<sub>2</sub> with rotation. In some embodiments, the tumors are digested overnight with constant rotation. In some embodiments, the tumors are digested overnight at 37°C, 5% CO<sub>2</sub> with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[00145] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00146] 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.

[00147] In some embodiments, the enzyme mixture comprises DNase. In some embodiments, the working stock for the DNase is a 10,000 IU/ml 10X working stock.

[00148] 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.

[00149] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml DNase, and 1 mg/ml hyaluronidase.

[00150] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml DNase, and 1 mg/ml hyaluronidase.

[00151] In some embodiments, the enzyme mixture comprises neutral protease. In some embodiments, the working stock for the neutral protease is reconstituted at a concentration of 175 DMC U/mL.

[00152] In some embodiments, the enzyme mixture comprises neutral protease, dNase, and collagenase.

[00153] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml dNase, and 0.31 DMC U/ml neutral protease. In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml dNase, and 0.31 DMC U/ml neutral protease.

[00154] In general, the harvested cell suspension is called a “primary cell population” or a “freshly harvested” cell population.

[00155] 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 prior to gene-editing.

[00156] In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained. In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 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  $27 \text{ mm}^3$ . In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about  $1300 \text{ mm}^3$  to about  $1500 \text{ mm}^3$ . In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about  $1350 \text{ mm}^3$ . 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. In some embodiments, the multiple fragments comprise about to about 100 fragments.

[00157] 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  $1 \text{ mm}^3$  and  $10 \text{ mm}^3$ . In some embodiments, the tumor fragment is between about  $1 \text{ mm}^3$  and  $8 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $1 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $2 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $3 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $4 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $5 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $6 \text{ mm}^3$ . In some embodiments, the tumor fragment

is about 7 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 8 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 9 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 10 mm<sup>3</sup>. In some embodiments, the tumors are 1-4 mm x 1-4 mm x 1-4 mm. In some embodiments, the tumors are 1 mm x 1 mm x 1 mm. In some embodiments, the tumors are 2 mm x 2 mm x 2 mm. In some embodiments, the tumors are 3 mm x 3 mm x 3 mm. In some embodiments, the tumors are 4 mm x 4 mm x 4 mm.

**[00158]** In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of fatty tissue on each piece.

**[00159]** In some embodiments, the tumor fragmentation is performed in order to maintain the tumor internal structure. In some embodiments, the tumor fragmentation is performed without preforming a sawing motion with a scalpel. In some embodiments, the TILs are obtained from tumor digests. In some embodiments, tumor digests were generated by incubation in enzyme media, for example but not limited to RPMI 1640, 2 mM GlutaMAX, 10 mg/mL gentamicin, 30 U/mL dNase, and 1.0 mg/mL collagenase, followed by mechanical dissociation (GentleMACS, Miltenyi Biotec, Auburn, CA). After placing the tumor in enzyme media, the tumor can be mechanically dissociated for approximately 1 minute. The solution can then be incubated for 30 minutes at 37 °C in 5% CO<sub>2</sub> and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% CO<sub>2</sub>, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% CO<sub>2</sub>. 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.

**[00160]** In some embodiments, the harvested cell suspension prior to the first expansion step is called a “primary cell population” or a “freshly harvested” cell population.

[00161] In some embodiments, cells can be optionally frozen after sample harvest and stored frozen prior to entry into the expansion described in further detail below.

[00162] In some embodiments, the TILs are obtained by thawing a cryopreserved tumor digest comprising a first population of TILs from a tumor resected from a subject. In some embodiments, the first population of TILs is obtained from a tumor resected from a subject by processing a tumor sample obtained from the subject into a tumor digest. In some embodiments, the tumor digest or tumor fragments comprise a first population of TILs and are obtained from a tumor that was resected from the subject.

[00163] In some embodiments, the methods disclosed herein comprise adding a tumor digest or tumor fragments into a closed system, wherein the tumor digest or tumor fragments comprise a first population of TILs and are obtained from a tumor that was resected from the subject. In some embodiments, the methods disclosed herein comprise performing a first expansion by thawing a cryopreserved tumor digest comprising a first population of TILs from a tumor that was resected from the subject. In some embodiments, the methods disclosed herein comprise obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into a tumor digest or multiple tumor fragments.

[00164] In some embodiments, a tumor lysate can be further obtained from the tumor digest through several freeze-thaw cycles or mass spectrometry procedures.

[00165] 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 priming step.

[00166] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00167] 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.

[00168] 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.

[00169] 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.

[00170] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 1000 IU/mL DNase, and 1 mg/mL hyaluronidase.

[00171] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 500 IU/mL DNase, and 1 mg/mL hyaluronidase.

[00172] 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.

[00173] In some embodiments, the TILs are not obtained from tumor digests. In some embodiments, the solid tumor cores are not fragmented.

[00174] In some embodiments, obtaining the first population of TILs comprises a multilesional sampling method.

[00175] 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.

[00176] 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).

[00177] In some instances, collagenase (such as animal free- type I 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.

**[00178]** 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.

**[00179]** 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.

**[00180]** 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.

**[00181]** In some embodiments, the enzyme mixture includes about 10.2- $\mu$ L of neutral protease (0.36 DMC U/mL), 21.3- $\mu$ L of collagenase (1.2 PZ/mL) and 250- $\mu$ L of DNase I (200 U/mL) in about 4.7 mL of sterile HBSS.

### **C. First Expansion**

**[00182]** After dissection or digestion of 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 tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 3 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, this primary

cell population is cultured for a period of 7 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 10 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of about 11 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells.

**[00183]** In some embodiments, expansion of TILs may be performed using an initial bulk TIL expansion step (which can include processes referred to as pre-REP) as described below and herein, followed by a second expansion (including processes referred to as rapid expansion protocol (REP) steps) as described below and herein, followed by optional cryopreservation, and followed by a second REP steps as described below and herein. The TILs obtained from this process may be optionally characterized for phenotypic characteristics and metabolic parameters as described herein.

**[00184]** In some embodiments where TIL cultures are initiated in 24-well plates, for example, using Costar 24-well cell culture cluster, flat bottom (Corning Incorporated, Coming, NY, each well can be seeded with  $1 \times 10^6$  tumor digest cells or one tumor fragment in 2 mL of complete medium (CM) with IL-2 (6000 IU/mL; Chiron Corp., Emeryville, CA). In some embodiments, the tumor fragment is between about  $1 \text{ mm}^3$  and  $10 \text{ mm}^3$ .

**[00185]** In some embodiments, the first expansion culture medium is referred to as “CM”, an abbreviation for culture media. In some embodiments, CM for Step B consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a  $10 \text{ cm}^2$  gas-permeable silicon bottom (for example, G-REX10; Wilson Wolf Manufacturing, New Brighton, MN), each flask was loaded with  $10\text{--}40 \times 10^6$  viable tumor digest cells or 5–30 tumor fragments in 10–40 mL of CM with IL-2. Both the G-REX10 and 24-well plates were incubated in a humidified incubator at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days.

**[00186]** 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.

**[00187]** 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00188]** 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<sup>+</sup>, Al<sup>3+</sup>, Ba<sup>2+</sup>, Cd<sup>2+</sup>, CO<sub>2</sub><sup>+</sup>, Cr<sup>3+</sup>, Ge<sup>4+</sup>, Se<sup>4+</sup>, Br, T, Mn<sup>2+</sup>, P, Si<sup>4+</sup>, V<sup>5+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, Rb<sup>+</sup>, Sn<sup>2+</sup> and Zr<sup>4+</sup>. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

**[00189]** In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00190]** 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.

**[00191]** In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55μM.

**[00192]** In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-

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

**[00193]** In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or

defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

**[00194]** In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55μM.

**[00195]** 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<sup>+</sup>, Al<sup>3+</sup>, Ba<sup>2+</sup>, Cd<sup>2+</sup>, CO<sub>2</sub><sup>+</sup>, Cr<sup>3+</sup>, Ge<sup>4+</sup>, Se<sup>4+</sup>, Br, T, Mn<sup>2+</sup>, P, Si<sup>4+</sup>, V<sup>5+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, Rb<sup>+</sup>, Sn<sup>2+</sup> and Zr<sup>4+</sup>. 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00196]** 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.

**[00197]** 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 4 below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading "A Preferred Embodiment of the 1X Medium" in Table 4. 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 11 above.

**[00198]** 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  $\mu$ M), 2-mercaptoethanol (final concentration of about 100  $\mu$ M).

**[00199]** 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.

**[00200]** 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  $\beta$ ME; also known as 2-mercaptoethanol, CAS 60-24-2).

**[00201]** After preparation of the tumor fragments, the resulting cells (i.e., fragments) are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum (or, in some cases, as outlined herein, in the presence of an APC cell population) with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 10 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, the growth media during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the IL is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of  $20\text{--}30 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of  $20 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of  $25 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of  $30 \times 10^6$  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  $6 \times 10^6$  IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepared as described in Example 5. In some embodiments, the first expansion culture media comprises about 10,000 IU/mL of IL-2, about 9,000 IU/mL of IL-2, about 8,000 IU/mL of IL-2, about 7,000 IU/mL of IL-2, about 6000 IU/mL of IL-2 or about 5,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 9,000 IU/mL of IL-2 to

about 5,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 8,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 7,000 IU/mL of IL-2 to about 6,000 IU/mL of IL-2. In some embodiments, the first expansion culture media comprises about 6,000 IU/mL of IL-2. In 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 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 about 8000 IU/mL of IL-2.

**[00202]** In some embodiments, first expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In 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.

**[00203]** In some embodiments, first expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-

21. In some embodiments, the first expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In 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.

**[00204]** In some embodiments, the cell culture medium comprises an anti-CD3 agonist antibody, e.g. 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 does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab. See, for example, Table 1.

**[00205]** 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.



[00206] 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.

[00207] In some embodiments, the first expansion culture medium is referred to as “CM”, an abbreviation for culture media. In some embodiments, it is referred to as CM1 (culture medium 1). In some embodiments, CM consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10cm<sup>2</sup> gas-permeable silicon bottom (for example, G-REX10; Wilson Wolf Manufacturing, New Brighton, MN), each flask was loaded with 10–40x10<sup>6</sup> viable tumor digest cells or 5–30 tumor fragments in 10–40mL of CM with IL-2. Both the G-REX10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% CO<sub>2</sub> and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days. In some embodiments, the CM is the CM1 described in the Examples, see, Example 1. In some embodiments, the first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the initial cell culture medium or the first cell culture medium comprises IL-2.

[00208] In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 82, which can include those sometimes referred to as the pre-REP) process is shortened to 3-14 days, as discussed in the examples and figures. In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 82, which can include those sometimes referred to as the pre-REP) is shortened to 7 to 14 days, as including for example, an expansion as described in Step B of Figure 82. In some embodiments, the first expansion of Step B is shortened to 10-14 days. In some embodiments, the first expansion is shortened to 11 days, as discussed in, for example, an expansion as described in Step B of Figure 82.

[00209] In some embodiments, the first TIL expansion can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the first TIL expansion can proceed for 1 day to 14 days. In some embodiments, the first TIL expansion can proceed for 2 days to 14 days. In some embodiments, the first TIL expansion can proceed for 3 days to 14 days. In some

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

**[00210]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first expansion, including for example during a Step B processes according to Figure 82, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 82 and as described herein.

**[00211]** In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 82) process is shortened to 3 to 14 days, as discussed in the examples and figures. In some embodiments, the first expansion of Step B is shortened to 7 to 14 days. In some embodiments, the first expansion of Step B is

shortened to 10 to 14 days. In some embodiments, the first expansion is shortened to 11 days. In some embodiments, the first expansion, for example, Step B according to Figure 82, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

#### **1. Cytokines and Other Additives**

[00212] 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.

[00213] Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is 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, or 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.

[00214] 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 other embodiments, 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.

#### **D. Second Expansion**

[00215] In some embodiments, the TIL cell population is expanded in number after initial bulk processing and pre-REP expansion to produce the population of TILs. This further expansion is referred to herein as the second expansion, which can include expansion

processes generally referred to in the art as a rapid expansion process (REP). The second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 agonist antibody, in a gas-permeable container.

[00216] In some embodiments, the 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, wherein the expanded TILs have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, the second expansion can proceed for 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the second expansion can proceed for about 7 days to about 14 days. In some embodiments, the second expansion can proceed for about 7 days to about 12 days. In some embodiments, the second expansion can proceed for about 7 days to about 10 days. In some embodiments, the second expansion can proceed for about 7 days to about 9 days. In some embodiments, the second expansion can proceed for about 8 days to about 9 days. In some embodiments, the second expansion can proceed for about 9 days. In some embodiments, the second expansion can proceed for about 10 days. In some embodiments, the second expansion can proceed for about 11 days.

[00217] In some embodiments, the second expansion can be performed in a gas permeable container using the methods of the present disclosure (including for example, expansions referred to as REP). 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 agonist 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  $\mu$ 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.

**[00218]** 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, or 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 does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

**[00219]** 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 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 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.

**[00220]** In some embodiments, REP and/or the second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. Media replacement is done (generally 2/3 media replacement via respiration with fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

[00221] In some embodiments, the second expansion (which can include processes referred to as the REP process) is shortened to 7-14 days, as discussed in the examples and figures, wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, the second expansion is shortened to 9 days.

[00222] In some embodiments, REP and/or the second expansion may be performed using T-175 flasks and gas permeable bags as previously described (Tran, *et al.*, *J. Immunother.* **2008**, *31*, 742-51; Dudley, *et al.*, *J. Immunother.* **2003**, *26*, 332-42) or gas permeable cultureware (G-Rex flasks), wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, the second expansion (including expansions referred to as rapid expansions) is performed in T-175 flasks, and about  $1 \times 10^6$  TILs suspended in 150 mL of media may be added to each T-175 flask. The TILs may be cultured in a 1 to 1 mixture of CM and AIM-V medium, supplemented with 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3. The T-175 flasks may be incubated at 37° C in 5% CO<sub>2</sub>, wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. Half the media may be exchanged on day 5 using 50/50 medium with 3000 IU per mL of IL-2. In some embodiments, on day 7 cells from two T-175 flasks may be combined in a 3 L bag and 300 mL of AIM V with 5% human AB serum and 3000 IU per mL of IL-2 was added to the 300 mL of TIL suspension. The number of cells in each bag was counted every day or two and fresh media was added to keep the cell count between  $0.5$  and  $2.0 \times 10^6$  cells/mL.

[00223] 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),  $5 \times 10^6$  or  $10 \times 10^6$  TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3), wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. The G-Rex 100 flasks may be incubated at 37°C in 5% CO<sub>2</sub>. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and centrifuged at 1500 rpm ( $491 \times g$ ) for 10 minutes. The TIL

pellets may be re-suspended with 150 mL of fresh medium with 5% human AB serum, 3000 IU per mL of IL-2, and added back to the original G-Rex 100 flasks. When TIL are expanded serially in G-Rex 100 flasks, on day 7 the TIL in each G-Rex 100 may be suspended in the 300 mL of media present in each flask and the cell suspension may be divided into 3 100 mL aliquots that may be used to seed 3 G-Rex 100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU per mL of IL-2 may be added to each flask. The G-Rex 100 flasks may be incubated at 37° C in 5% CO<sub>2</sub> and after 4 days 150 mL of AIM-V with 3000 IU per mL of IL-2 may be added to each G-REX 100 flask. The cells may be harvested on day 14 of culture.

**[00224]** 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),  $5 \times 10^6$  or  $10 \times 10^6$  TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3). The G-REX-100 (or G-REX100M) flasks may be incubated at 37°C in 5% CO<sub>2</sub>. 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 TILs 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 (or G-REX500M). 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. In some embodiments, the process employed varying centrifugation speeds (400g, 300g, 200g for 5 minutes) and varying numbers of repetitions.

**[00225]** In some embodiments, the second expansion (including expansions referred to as REP) is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. In some embodiments, media replacement is done until the cells are transferred to an

alternative growth chamber, wherein the TILs expanded by such a second expansion have been transduced with the recombinant lentiviral particle disclosed herein to produce the population of gene-edited TILs. In some embodiments, 2/3 of the media is replaced by aspiration of spent media followed by infusion with fresh media. In some embodiments, alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

[00226] In some embodiments, the second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below.

[00227] 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.

[00228] 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.

[00229] 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ , Br, T,  $\text{Mn}^{2+}$ , P,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

**[00230]** In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™OpTmizer™ T-cell Expansion Basal Medium, CTS™OpTmizer™ T-cell Expansion SFM, CTS™AIM-V Medium, CST™AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00231]** 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.

**[00232]** In some embodiments, the serum-free or defined medium is CTS™OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™OpTmizer™ is useful in the present invention. CTS™OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™Immune Cell Serum

Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55 $\mu$ M.

**[00233]** In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 6000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific)

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

**[00234]** In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

**[00235]** In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55µM.

**[00236]** 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ , Br, T,  $\text{Mn}^{2+}$ , P,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00237] 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.

**[00238]** 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 12 below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading “A Preferred Embodiment of the 1X Medium” in Table 12. 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 12 above.

**[00239]** 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  $\mu$ M), 2-mercaptoethanol (final concentration of about 100  $\mu$ M).

**[00240]** 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.

**[00241]** 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  $\beta$ BME; also known as 2-mercaptoethanol, CAS 60-24-2).

**[00242]** In some embodiments, the 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 single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

**[00243]** In some embodiments, the steps of the method are completed within a period of about 22 days. In some embodiments, the steps of the method are completed within a period of about 8 days. In some embodiments, the steps of the method are completed within a period of about 9 days. In some embodiments, the steps of the method are completed within a period of about 10 days. In some embodiments, the steps of the method are completed within a period of about 11 days. In some embodiments, the steps of the method are completed within a period of about 12 days. In some embodiments, the steps of the method are completed within a period of about 13 days. In some embodiments, the steps of the method are completed within a period of about 14 days. In some embodiments, the steps of the method are completed within a period of about 15 days. In some embodiments, the steps of the method are completed within a period of about 16 days. In some embodiments, the steps of the method are completed within a period of about 17 days. In some embodiments, the steps of the method are completed within a period of about 18 days. In some embodiments, the steps of the method are completed within a period of about 19 days. In some embodiments, the steps of the method are completed within a period of about 20 days. In some embodiments, the steps of the method are completed within a period of about 21 days. In some embodiments, the steps of the method are completed within a period of about 22 days. In some embodiments, the steps of the method are completed within a period of about 23 days. In some embodiments, the steps of the method are completed within a period of about 24 days. In some embodiments, the steps of the method are completed within a period of about 25 days. In some embodiments, the steps of the method are completed within a period of about 26 days. In some embodiments, the steps of the method are completed within a period of about 27 days. In some embodiments, the steps of the method are completed within a period of about 28 days. In some embodiments, the steps of the method are completed within a period of about 29 days. In some embodiments, the steps of the method are completed within a period of about 30 days. In some embodiments, the steps of the method are completed within a period of about 31 days.

**[00244]** In some embodiments, the antigen presenting cells (APCs) are PBMCs. According to some embodiments, the PBMCs are irradiated. According to some embodiments, the PBMCs are allogeneic. According to some embodiments, the PBMCs are irradiated and allogeneic. According to some embodiments, the antigen-presenting cells are artificial antigen-presenting cells.

[00245] In some embodiments, the first expansion is performed using a gas permeable container. In some embodiments, the second expansion is performed using a gas permeable container.

[00246] In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof. In some embodiments, the second cell culture medium and/or third culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

#### 1. Feeder Cells and Antigen Presenting Cells

[00247] In some embodiments, the second expansion procedures described herein require an excess of feeder cells during REP TIL expansion and/or during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

[00248] 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.

[00249] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

[00250] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody, IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.

[00251] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells,

cultured in the presence of OKT3, IL-15 and IL-21, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/mL OKT3 antibody, IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/mL OKT3 antibody, IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/mL OKT3 antibody, IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/mL OKT3 antibody, IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.

**[00252]** 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.

**[00253]** In some embodiments, the second expansion procedures described herein require a ratio of about  $2.5 \times 10^9$  feeder cells to about  $100 \times 10^6$  TIL. In other embodiments, the second expansion procedures described herein require a ratio of about  $2.5 \times 10^9$  feeder cells to about  $50 \times 10^6$  TIL. In yet other embodiments, the second expansion procedures described herein require about  $2.5 \times 10^9$  feeder cells to about  $25 \times 10^6$  TIL.

**[00254]** In some embodiments, the second expansion procedures described herein require an excess of feeder cells during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.



[00255] In some embodiments, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

## 2. Cytokines and Other Additives

[00256] 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.

[00257] Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILs is 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.

[00391] In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-2. In some embodiments, the IL-2 is at a concentration of 3000 IU/mL or lower. In some embodiments, the first cell culture medium or the second cell culture medium contains no added IL-2. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 1 ng/mL to about 100 ng/mL. In some embodiments, the first cell culture medium or the second cell culture medium comprises IL-15 and/or IL-21 at a concentration of about 10 ng/mL. In some embodiments, the first cell culture medium comprises IL-2 and IL-21. In some embodiments, the first cell culture medium comprises IL-2 at 3000 IU/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises IL-15 and IL-21. In some embodiments, the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL. In some embodiments, the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and a protein kinase B (AKT) inhibitor. In some embodiments, the AKT inhibitor is selected from the group consisting of ipatasertib, GSK690693, GSK2141795, GSK2110183, AZD5363, GDC-0068, AT7867, CCT128930, MK-2206, BAY 1125976, Perifosine, Oridonin, Herbacetin, Tehranolide, Isoliquiritigenin, Scutellarin, and Honokiol.

[00392] In some embodiments, the first expansion is performed over a period of about 7-11 days. In some embodiments, the second expansion is performed over a period of about 7-11 days.

### 3. Modifiers of T Cell Metabolism

[00393] In vitro expansion alters TIL cellular state, which correlates with ACT efficacy (Chiffelle, J., et al., *bioRxiv* **2023**, the content of which is hereby incorporated by reference in its entirety). Rapidly expanding TILs have bioenergetic and biosynthetic needs. Increasing the availability of essential cofactors such as L-arginine and NAD<sup>+</sup> may support synthesis of major biomass components. To functionally reinvigorate cells during in vitro expansion may produce TILs with renewed effector features.

[00394] Therefore, provided herein are modifiers of T cell metabolism that increase availability of essential cofactors L-arginine and NAD<sup>+</sup> to support synthesis of major biomass components in TILs, which may be added to the second expansion (REP) of TILs.

[00395] L-arginine, a building block for protein synthesis, improves T cell function via metabolic modification (Geiger R., et al., *Cell* **2016**: 167; 829-842; Fultang, L., *Blood* **2020**; 136: 1155-60; the contents of which are hereby incorporated by reference in their entireties).

[00396] NAD<sup>+</sup> (nicotinamide adenine dinucleotide), an electron acceptor, is heavily consumed during biomass synthesis, and supplementation improves T cell function (Canto C, et al., *Cell Metabolism* **2015**; 22:31-53; Wang Y., et al., *Cell Reports* **2021**; 36:1-12; the contents of which are hereby incorporated by reference in their entireties).

[00397] NAD<sup>+</sup> (Nicotinamide adenine dinucleotide) is a central coenzyme in cellular metabolism, particularly in redox reactions where it cycles between oxidized (NAD<sup>+</sup>) and reduced (NADH) states. In the context of TILs, NAD<sup>+</sup> plays a critical role in modulating their metabolic fitness and function. TILs operate in the tumor microenvironment, which is often characterized by nutrient scarcity and hypoxia. In this challenging microenvironment, TILs must adapt their metabolism to sustain their anti-tumor activity.

[00398] NAD<sup>+</sup> enhances the metabolic fitness of T cells in several ways:

[00399] Glycolysis and the Pentose Phosphate Pathway (PPP): Although glycolysis does not directly use NAD<sup>+</sup>, the regeneration of NAD<sup>+</sup> from NADH is critical to maintain glycolytic flux, which is particularly important in hypoxic conditions where mitochondrial respiration is limited. The PPP, which branches from glycolysis, relies on NADP<sup>+</sup> (the phosphorylated

form of NAD<sup>+</sup>) for the generation of ribose-5-phosphate and for the reduction of glutathione, contributing to nucleotide synthesis and antioxidant defense mechanisms.

**[00400]** Mitochondrial Function: NAD<sup>+</sup> is essential in the mitochondria as it is a key electron acceptor in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Enhanced mitochondrial function can support the differentiation and survival of memory T cells, which are crucial for sustained anti-tumor immunity.

**[00401]** Sirtuins Activation: Sirtuins are a family of NAD<sup>+</sup>-dependent deacetylases that regulate cellular metabolism, stress responses, and inflammation. By activating sirtuins, NAD<sup>+</sup> can promote the formation of memory T cells and enhance their anti-tumor function.

**[00402]** PARP Activity: NAD<sup>+</sup> is a substrate for poly(ADP-ribose) polymerases (PARPs) which are involved in DNA repair and genomic stability. By facilitating the function of PARPs, NAD<sup>+</sup> helps maintain TIL integrity in the face of DNA-damaging conditions within tumors.

**[00403]** To replicate and enhance the benefits provided by NAD<sup>+</sup> to TILs, both pharmacological and genetic approaches can be considered:

**[00404]** Pharmacologic Approaches:

- NAD<sup>+</sup> Precursors: Boosting NAD<sup>+</sup> via administration of NAD<sup>+</sup> precursors such as nicotinamide riboside (NR) or nicotinamide mononucleotide (NMN) can increase intracellular NAD<sup>+</sup> levels and thereby support TIL metabolism and function.
- NAD<sup>+</sup>: Boosting availability of NAD<sup>+</sup> metabolic substrate directly to TILs
- Sirtuin Activators: Compounds like resveratrol or SRT1720 can activate sirtuins, thus mimicking the effect of high NAD<sup>+</sup> levels and promoting TIL anti-tumor activity.
- PARP Inhibitors: While PARP activity is NAD<sup>+</sup> dependent and can be beneficial, excessive activation can deplete cellular NAD<sup>+</sup> reserves. PARP inhibitors can help preserve NAD<sup>+</sup> levels under stress conditions, potentially supporting T cell function.
- CD38 Inhibitors: CD38 uses NAD<sup>+</sup> to generate ADP-ribose and cyclic ADP-ribose, which are important for calcium signaling but also deplete NAD<sup>+</sup>. Inhibitors of CD38 may help preserve NAD<sup>+</sup> levels in TILs.

**[00405]** Genetic Approaches:

- Overexpression of NAMPT: This is the rate-limiting enzyme in the NAD<sup>+</sup> salvage pathway. Overexpressing NAMPT can boost the intracellular levels of NAD<sup>+</sup> in TILs.

- Manipulation of Sirtuin Genes: Genetic modification to overexpress sirtuin genes can potentially mimic the effect of high NAD<sup>+</sup> levels and enhance TIL function.
- Gene Silencing of NAD<sup>+</sup> Consuming Enzymes: Silencing or knocking down genes encoding for enzymes that heavily consume NAD<sup>+</sup>, like CD38 or PARP family members, might conserve NAD<sup>+</sup> for metabolic processes essential for TILs.
- TCA Cycle and OXPHOS Support: Enhancing genes involved in mitochondrial biogenesis and function can support the efficient use of NAD<sup>+</sup> and maintain TIL metabolic fitness.

[00406] Pharmacologic and genetic tools to augment NAD<sup>+</sup> utilization in TILs center on specific molecular targets and pathways. In some embodiments, exemplary tools for augment NAD<sup>+</sup> utilization focus on one or more of the following functions: mitochondrial biogenesis, promoting effective energy utilization, and fostering an anti-tumor phenotype. In embodiments, such tools are implemented in a concerted manner, with attention to the balance between enhancing TIL function and avoiding over-stimulation that could lead to exhaustion or apoptosis. In some embodiments, the heterogeneity within the TIL population and the unique microenvironment of each tumor when designing these interventions are taken into consideration. The modulation of mitochondrial function through pharmacological and genetic approaches could thus be a powerful strategy to enhance the efficacy of adoptive TIL therapies. Tools for augmenting NAD<sup>+</sup> utilization are further detailed below.

#### **Pharmacological Manipulation of NAD<sup>+</sup> Pathways:**

##### **6. NAD<sup>+</sup> Precursors and NAD<sup>+</sup>:**

- o **Nicotinamide Riboside (NR)** and **Nicotinamide Mononucleotide (NMN)** are NAD<sup>+</sup> precursors. They are converted to NAD<sup>+</sup> via the salvage pathway involving the enzyme nicotinamide riboside kinase (NRK1/2) for NR and NMN adenylyltransferase (NMNAT1/2/3) for NMN.
- o **Nicotinic Acid (NA)** can also be used, which is converted into NAD<sup>+</sup> via the Preiss-Handler pathway, although it can cause flushing due to prostaglandin-mediated vasodilation.
- o **Nicotinamide dinucleotide (NAD<sup>+</sup>)** itself can also be used, as it has been shown to cross the lipid bilayer and enter the mitochondria.

##### **7. Sirtuin Modulators:**

- **Resveratrol** is a sirtuin-activating compound (STAC) that enhances SIRT1 activity, which in turn can deacetylate and activate peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ), a master regulator of mitochondrial biogenesis.
- **SRT1720**, another STAC, has been shown to selectively activate SIRT1, leading to enhanced mitochondrial function and possible improvement in TIL survival and function.

#### 8. **PARP Inhibitors:**

- Compounds like **Olaparib** and **Niraparib** can inhibit PARP enzymes, thus conserving NAD<sup>+</sup> for other metabolic processes critical for T cell function and potentially reducing the metabolic competition between PARPs and SIRT1 for NAD<sup>+</sup>.

#### 9. **CD38 Inhibitors:**

- **Isatuximab** and **Daratumumab** are monoclonal antibodies that target CD38, which is responsible for NAD<sup>+</sup> degradation. Small molecule inhibitors of CD38 are also in development and could be repurposed to enhance NAD<sup>+</sup> levels in TILs.

#### 10. **Mitochondrial Metabolic Modulators:**

- **Metformin** has been shown to activate AMP-activated protein kinase (AMPK), which in turn activates PGC-1 $\alpha$ , improving mitochondrial biogenesis and potentially restoring TIL function.
- **Pioglitazone**, a PPAR $\gamma$  agonist, might also enhance mitochondrial biogenesis and function in TILs through PGC-1 $\alpha$  induction.
- **Bezafibrate**: A pan-PPAR agonist that can induce PGC-1 $\alpha$ , enhancing mitochondrial function and fatty acid oxidation

### **Mitochondrial Biogenesis and Dynamics:**

#### 3. **PGC-1 $\alpha$ Activation:**

- Specific activators of PGC-1 $\alpha$  or direct overexpression via gene delivery systems could be used to stimulate mitochondrial biogenesis.

#### 4. **Mitochondrial Dynamics:**

- Manipulating genes involved in mitochondrial fusion (e.g., **MFN1/2**, **OPA1**) and fission (e.g., **DRP1**, **FIS1**) to maintain mitochondrial network integrity and function.

**Genetic Tools:****9. Overexpression of NAD<sup>+</sup> Synthesis Genes:**

- *NAMPT*: Overexpression can increase the salvage pathway flux, boosting NAD<sup>+</sup> levels.
- *NMNAT1/2/3*: Increased expression of NMN adenylyltransferases to catalyze the conversion of NMN to NAD<sup>+</sup>.

**10. Sirtuin Gene Manipulation:**

- *SIRT1-7*: Overexpressing Sirtuin genes, with a focus on SIRT1 for its role in mitochondrial biogenesis, SIRT3 for mitochondrial protein deacetylation, and SIRT6 for maintaining genomic stability.

**11. Upregulation of TCA Cycle and OXPHOS Genes:**

- *PC (Pyruvate Carboxylase)*: Enhancing anaplerotic reactions to replenish TCA cycle intermediates.
- *PDK (Pyruvate Dehydrogenase Kinase) Inhibitors*: Genetic silencing of PDK to increase PDH activity and promote glucose oxidation.

**12. Downregulation of NAD<sup>+</sup> Consuming Enzymes:**

- Using CRISPR/Cas9 or siRNA approaches to knockdown CD38 or PARP family members to conserve NAD<sup>+</sup> for enhancing TIL function.

**13. Promotion of Mitochondrial DNA Stability and Biogenesis:**

- *TFAM (Mitochondrial Transcription Factor A)*: Overexpression can promote mitochondrial DNA replication and transcription, thus enhancing mitochondrial function.
- *OGG1 or MUTYH*: Overexpression to enhance base-excision repair and maintain mitochondrial DNA integrity.

**14. Redox Balance:**

- *GCLC/GCLM (Glutamate-Cysteine Ligase Catalytic and Modifier Subunit)*: Overexpression to increase glutathione synthesis, a key antioxidant within mitochondria.

**15. Metabolic Reprogramming:**

- *CPT1A (Carnitine Palmitoyltransferase 1A)*: Genetic modification to boost fatty acid oxidation, which could sustain TIL function under nutrient-deprived conditions.

**16. Mitophagy and Autophagy Regulators:**

- *PRKN* (*Parkin*) and *PINK1*: Enhance mitophagy to remove dysfunctional mitochondria.
- *ATG5* or *ATG7*: Modulation of autophagy-related genes to balance energy and organelle quality control in TILs.

**[00407]** In some embodiments, these strategies may be optimized for dose, timing, and delivery mechanisms to ensure the TILs are metabolically enhanced without inducing detrimental effects such as apoptosis or senescence. In embodiments, such strategies are also assessed to avoid the promotion of tumor cell survival or immune evasion. In some embodiments, a combination of these strategies, informed by the specific metabolic deficiencies observed in the TILs isolated from individual tumors, provides a potent and personalized enhancement of TIL function for adoptive cell therapies.

| Method/Compound                   | Mechanism of Action                                                                   | Target Process/Pathway                | Potential Use in TILs                                     | Considerations/Challenges                          |
|-----------------------------------|---------------------------------------------------------------------------------------|---------------------------------------|-----------------------------------------------------------|----------------------------------------------------|
| Nicotinamide Riboside (NR)        | Precursor to NAD <sup>+</sup> ; converted to NMN and then to NAD <sup>+</sup>         | NAD <sup>+</sup> salvage pathway      | Enhance TIL metabolism and function                       | Dosage optimization to avoid side effects          |
| Nicotinamide Mononucleotide (NMN) | Direct precursor to NAD <sup>+</sup> ; converted to NAD <sup>+</sup> by NMNAT enzymes | NAD <sup>+</sup> salvage pathway      | Improve TIL anti-tumor activity                           | Stability and cellular uptake considerations       |
| Niacin (Vitamin B3)               | Converted to NAD <sup>+</sup> via the Preiss-Handler pathway                          | NAD <sup>+</sup> biosynthesis pathway | Support TIL energy generation and DNA repair              | Flushing reaction at high doses                    |
| NAMPT Inhibitors                  | Increase NAD <sup>+</sup> levels by inhibiting its degradation                        | NAD <sup>+</sup> salvage pathway      | Potentially extend TIL lifespan                           | Risk of systemic NAD <sup>+</sup> depletion        |
| PARP Inhibitors                   | Prevent NAD <sup>+</sup> depletion by inhibiting PARP enzymes                         | DNA repair pathway                    | Preserve NAD <sup>+</sup> for metabolic functions in TILs | Balance between inhibiting PARP and cancer therapy |



| Method/Compound            | Mechanism of Action                                                    | Target Process/Pathway                                  | Potential Use in TILs                               | Considerations/Challenges                          |
|----------------------------|------------------------------------------------------------------------|---------------------------------------------------------|-----------------------------------------------------|----------------------------------------------------|
| Sirtuin Activators         | Increase NAD <sup>+</sup> utilization efficiency                       | Sirtuin-mediated deacetylation                          | Enhance TIL mitochondrial function                  | Long-term effects on TILs are not fully understood |
| CD38 Inhibitors            | Reduce NAD <sup>+</sup> degradation                                    | ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase pathway | Increase NAD <sup>+</sup> bioavailability for TILs  | May affect calcium signaling                       |
| Ketogenic Diet             | May elevate NAD <sup>+</sup> levels via increased fatty acid oxidation | Metabolic shift to ketone body usage                    | Could indirectly support TILs in vivo               | Feasibility in patients and impact on immune cells |
| Caloric Restriction        | Can upregulate NAD <sup>+</sup> biosynthesis pathways                  | General metabolic downregulation                        | Potentially increase TIL efficacy                   | Patient compliance and nutritional concerns        |
| Exercise                   | Upregulates NAD <sup>+</sup> biosynthesis and salvage pathways         | Enhanced muscle contraction and energy demand           | Indirect systemic effects on immune function        | Direct impact on TILs is unclear                   |
| NAD <sup>+</sup> Infusions | Direct supplementation of NAD <sup>+</sup>                             | Bypasses NAD <sup>+</sup> biosynthesis pathways         | Immediate replenishment of NAD <sup>+</sup> in TILs | Risk of imbalances and homeostasis disruption      |

| Method/Compound | Mechanism of Action                                     | Target Process/Pathway                              | Potential Use in TILs                             | Considerations/Challenges                      |
|-----------------|---------------------------------------------------------|-----------------------------------------------------|---------------------------------------------------|------------------------------------------------|
| Gene Therapy    | Overexpression of NAD <sup>+</sup> biosynthesis enzymes | Targeted enhancement of NAD <sup>+</sup> production | Sustained increase in TIL NAD <sup>+</sup> levels | Vector design, delivery, and integration risks |

[00408] The evolutionary conserved Wnt/ $\beta$ -catenin pathway is critically involved in pluripotency and differentiation of embryonic stem cells via maintenance of DNA methylation patterns promoting epigenetic stability necessary for correct cell fate decisions during states of high cell division (Clevers, H. and R. Nusse, *Cell*, **2012**. 149(6): p. 1192-205). In humans, rapid recall of memory T cells upon re-exposure to antigen suggests the requirement of a stable epigenetic program. Most TIL-based ACT protocols for solid tumors are generated from terminally differentiated effector memory T cells resulting from repetitive rounds of TCR ligation during the ex vivo phase (Hinrichs, C.S. and S.A. Rosenberg, *Immunol Rev*, **2014**. 257(1): p. 56-71). However, the critical involvement of DNA methylation in shaping expansion, memory state status and thus functionality of antigen-specific TILs undergoing TCR ligation-dependent expansion ex vivo is not known (Abdelsamed, H.A., et al., *J Exp Med*, **2017**. 214(6): p. 1593-1606).

[00409] There is evidence to show that pharmacological activation of the Wnt/ $\beta$ -catenin signaling reprograms tumor-specific CD8<sup>+</sup> TILs in tumor-bearing mice by increasing expression of Tcf1 (encoded by Tcf7) (Gattinoni, L., et al., *Nat Med*, **2009**. 15(7): p. 808-13). In human melanoma, CD8<sup>+</sup>TCF7<sup>+</sup> uncovers a subset of TILs with a memory precursor-like cell state with bystander cytotoxic function and is associated with positive outcome to immune checkpoint blockade (Li, H., et al., *Cell*, **2019**. 176(4): p. 775-789 e18; Sade-Feldman, M., et al., *Cell*, **2018**. 175(4): p. 998-1013 e20). Downregulation of the components of the Wnt pathway has been reported in PD1<sup>+</sup> CD8<sup>+</sup> memory TILs in vivo and a further downregulation upon *in vitro expansion* in all T cell populations (Lipp, J.J., et al., *Oncoimmunology*, **2022**. 11(1): p. 2019466), whereas compensating the lack of Wnt signaling through pharmacological inhibition of GSK3 $\beta$  improves the effector function of in vitro expanded populations. As Wnt signaling is known to arrest the development of effector

CD8<sup>+</sup> T cells, it also might be involved in epigenetically reprogramming TILs. GSK-3 is a positive regulator of PD-1 expression in CD8<sup>+</sup> T cells and GSK-3 inhibition enhances T cell function, and is effective in controlling tumor growth (Taylor, A., et al., *Immunity*, **2016**, 44(2): p. 274-86; Steele, L., et al., *iScience*, **2021**, 24(6): p. 102555). Besides being a central regulator of PD-1, GSK-3 also negatively regulates Lymphocyte Activation Gene-3 (LAG-3) expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells and small molecule inhibitors of GSK-3 were more effective than LAG-3 blockade alone in suppressing tumor growth in murine model of melanoma (Rudd, C.E., et al., *Cell Rep*, **2020**, 30(7): p. 2075-2082 e4). More recently, it was demonstrated that Wnt activation promotes human memory T cell polyfunctionality via epigenetic regulator PRMT1 (Sung, B.Y., et al., *J Clin Invest*, **2022**, 132(2)).

[00410] Therefore, in some embodiments, the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ . In some embodiments, the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.

#### E. Harvest TILs

[00258] After the second expansion step, cells can be harvested. TILs can be harvested in any appropriate and sterile manner, including for example by centrifugation. Methods for TIL harvesting are well known in the art and any such know methods can be employed with the present process. In some embodiments, TILs are harvested using an automated system.

[00259] Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing systems is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term "LOVO cell processing system" also refers to any instrument or device manufactured by any vendor that can pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

[00260] In some embodiments, the harvest is performed from a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

[00261] In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in the Examples is employed.

#### **F. Final Formulation and Transfer to Infusion Container**

[00761] After the steps as outlined in detailed above and herein are complete, 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, such as an infusion bag, for use in administration to a patient. 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.

[00762] 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.

[00262] 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.

**[00263]** In some embodiments, TILs 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 by methods described in 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.

#### **G. Closed Systems for TIL Manufacturing**

**[00264]** The present invention provides for the use of closed systems during the TIL culturing process. Such closed systems allow for preventing and/or reducing microbial contamination, allow for the use of fewer flasks, and allow for cost reductions. In some embodiments, the closed system uses two containers.

**[00265]** Such closed systems are well-known in the art and can be found, for example, at <http://www.fda.gov/cber/guidelines.htm> and <https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076779.htm>.

**[00266]** Sterile connecting devices (STCDs) produce sterile welds between two pieces of compatible tubing. This procedure permits sterile connection of a variety of containers and tube diameters. In some embodiments, the closed systems include luer lock and heat-sealed systems as described in the Examples. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in the examples is employed. In some embodiments, the TILs are formulated into a final product formulation container according to the methods described herein in the examples.

**[00267]** In some embodiments, the closed system uses one container from the time the tumor fragments are obtained until the TILs are ready for administration to the patient or cryopreserving. In some embodiments when two containers are used, the first container is a closed G-container (such as G-rex100M series or G-rex500M series flasks) and the population of TILs is centrifuged and transferred to an infusion bag without opening the first

closed G-container. In some embodiments, when two containers are used, the infusion bag is a HypoThermosol-containing infusion bag. A closed system or closed TIL cell culture system is characterized in that once the tumor sample and/or tumor fragments have been added, the system is tightly sealed from the outside to form a closed environment free from the invasion of bacteria, fungi, and/or any other microbial contamination.

**[00268]** In some embodiments, the reduction in microbial contamination is between about 5% and about 100%. In some embodiments, the reduction in microbial contamination is between about 5% and about 95%. In some embodiments, the reduction in microbial contamination is between about 5% and about 90%. In some embodiments, the reduction in microbial contamination is between about 10% and about 90%. In some embodiments, the reduction in microbial contamination is between about 15% and about 85%. In some embodiments, the reduction in microbial contamination is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or about 100%.

**[00269]** The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

**[00270]** Moreover, pH, carbon dioxide partial pressure and oxygen partial pressure of the TIL cell culture environment each vary as the cells are cultured. Consequently, even though a medium appropriate for cell culture is circulated, the closed environment still needs to be constantly maintained as an optimal environment for TIL proliferation. To this end, it is desirable that the physical factors of pH, carbon dioxide partial pressure and oxygen partial pressure within the culture liquid of the closed environment be monitored by means of a sensor, the signal whereof is used to control a gas exchanger installed at the inlet of the culture environment, and the that gas partial pressure of the closed environment be adjusted in real time according to changes in the culture liquid so as to optimize the cell culture environment. In some embodiments, the present invention provides a closed cell culture system which incorporates at the inlet to the closed environment a gas exchanger equipped with a monitoring device which measures the pH, carbon dioxide partial pressure and oxygen partial pressure of the closed environment, and optimizes the cell culture environment by automatically adjusting gas concentrations based on signals from the monitoring device.

[00271] In some embodiments, the pressure within the closed environment is continuously or intermittently controlled. That is, the pressure in the closed environment can be varied by means of a pressure maintenance device for example, thus ensuring that the space is suitable for growth of TILs in a positive pressure state, or promoting exudation of fluid in a negative pressure state and thus promoting cell proliferation. By applying negative pressure intermittently, moreover, it is possible to uniformly and efficiently replace the circulating liquid in the closed environment by means of a temporary shrinkage in the volume of the closed environment.

[00272] In some embodiments, additional equipment, such as an electroporator (e.g., a Neon electroporator) is a component of an all-closed system. In some embodiments, optimal culture components for proliferation of the TILs can be substituted or added, and including factors such as IL-2 and/or OKT3, as well as combination, can be added.

[00273] In other embodiments, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-10. In other embodiments, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-100M. In other embodiments, the invention provides the method described in any of the preceding paragraphs as applicable above modified such that each container recited in the method is a GREX-500M.

#### **VIII. Gen 2 TIL Manufacturing Processes**

[00274] An exemplary family of TIL processes known as Gen 2 (also known as process 2A) containing some of these features is depicted in Figures 82 and 83. An embodiment of Gen 2 is shown in Figure 83.

[00275] As discussed herein, the present invention can include a step relating to the restimulation of cryopreserved TILs to increase their metabolic activity and thus relative health prior to transplant into a patient, and methods of testing said metabolic health. As generally outlined herein, TILs are generally taken from a patient sample and manipulated to expand their number prior to transplant into a patient. In some embodiments, the TILs may be optionally genetically manipulated as discussed below.

[00276] In some embodiments, the TILs may be cryopreserved. Once thawed, they may also be restimulated to increase their metabolism prior to infusion into a patient.

[00277] In some embodiments, the first expansion (including processes referred to as the pre-REP as well as processes shown in Figure 82 as Step A) is shortened to 3 to 14 days and the second expansion (including processes referred to as the REP as well as processes shown in Figure 82 as Step B) is shortened to 7 to 14 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the first expansion (for example, an expansion described as Step B in Figure 82) is shortened to 11 days and the second expansion (for example, an expansion as described in Step D in Figure 82) is shortened to 11 days. In some embodiments, the combination of the first expansion and second expansion (for example, expansions described as Step B and Step D in Figure 82) is shortened to 22 days, as discussed in detail below and in the examples and figures.

[00278] The “Step” Designations A, B, C, etc., below are in reference to Figure 82 and in reference to certain embodiments described herein. The ordering of the Steps below and in Figure 82 is exemplary and any combination or order of steps, as well as additional steps, repetition of steps, and/or omission of steps is contemplated by the present application and the methods disclosed herein.

#### **A. STEP A: Obtain Patient Tumor Sample**

[00279] In general, TILs are initially obtained from a patient tumor sample and then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, restimulated as outlined herein and optionally evaluated for phenotype and metabolic parameters as an indication of TIL health.

[00280] A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In some embodiments, multilesional sampling is used. In some embodiments, surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells includes multilesional sampling (i.e., obtaining samples from one or more tumor sites and/or locations in the patient, as well as one or more tumors in the same location or in close proximity). In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of lung tissue. In some embodiments, useful TILs are obtained from endometrial cancer. The



solid tumor may be of skin tissue. In some embodiments, useful TILs are obtained from a melanoma.

**[00281]** Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 mm<sup>3</sup>, with from about 2-3 mm<sup>3</sup> being particularly useful. In some embodiments, the TILs are cultured from these fragments using enzymatic tumor digests. Such tumor digests may be produced by incubation in enzymatic media (e.g., Roswell Park Memorial Institute (RPMI) 1640 buffer, 2 mM glutamate, 10 mcg/mL gentamicine, 30 units/mL of DNase and 1.0 mg/mL of collagenase) followed by mechanical dissociation (e.g., using a tissue dissociator). Tumor digests may be produced by placing the tumor in enzymatic media and mechanically dissociating the tumor for approximately 1 minute, followed by incubation for 30 minutes at 37 °C in 5% CO<sub>2</sub>, 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.

**[00282]** 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 (dispase), 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.

**[00283]** 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 HBSS.

**[00284]** 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 289.2 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.

**[00285]** 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.

**[00286]** 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-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.

**[00287]** In some embodiments, the stock of enzymes is variable and the concentrations may need to be determined. In some embodiments, the concentration of the lyophilized stock can be verified. In some embodiments, the final amount of enzyme added to the digest cocktail is adjusted based on the determined stock concentration.

**[00288]** In some embodiment, the enzyme mixture includes about 10.2- $\mu$ L of neutral protease (0.36 DMC U/mL), 21.3  $\mu$ L of collagenase (1.2 PZ/mL) and 250- $\mu$ L of DNase I (200 U/mL) in about 4.7 mL of sterile HBSS.

**[00289]** As indicated above, in some embodiments, the TILs are derived from solid tumors. In some embodiments, the solid tumors are not fragmented. In some embodiments, the solid tumors are not fragmented and are subjected to enzymatic digestion as whole

tumors. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and hyaluronidase. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% CO<sub>2</sub>. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% CO<sub>2</sub> with rotation. In some embodiments, the tumors are digested overnight with constant rotation. In some embodiments, the tumors are digested overnight at 37°C, 5% CO<sub>2</sub> with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[00290] In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and neutral protease. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and neutral protease for 1-2 hours. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and neutral protease for 1-2 hours at 37°C, 5% CO<sub>2</sub>. In some embodiments, the tumors are digested in an enzyme mixture comprising collagenase, DNase, and neutral protease for 1-2 hours at 37°C, 5% CO<sub>2</sub> with rotation. In some embodiments, the tumors are digested overnight with constant rotation. In some embodiments, the tumors are digested overnight at 37°C, 5% CO<sub>2</sub> with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[00291] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00292] 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.

[00293] In some embodiments, the enzyme mixture comprises DNase. In some embodiments, the working stock for the DNase is a 10,000 IU/mL 10X working stock.

[00294] 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.

[00295] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 1000 IU/mL DNase, and 1 mg/mL hyaluronidase.

[00296] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 500 IU/mL DNase, and 1 mg/mL hyaluronidase.

[00297] In general, the harvested cell suspension is called a “primary cell population” or a “freshly harvested” cell population.

[00298] 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 digesting or fragmenting a tumor sample obtained from a patient.

[00299] In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained in, for example, Step A (as provided in Figure 82). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about  $27 \text{ mm}^3$ . In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about  $1300 \text{ mm}^3$  to about  $1500 \text{ mm}^3$ . In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about  $1350 \text{ mm}^3$ . 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.

[00300] 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  $1 \text{ mm}^3$  and  $10 \text{ mm}^3$ . In some embodiments, the tumor fragment is between about  $1 \text{ mm}^3$  and  $8 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $1 \text{ mm}^3$ . In some embodiments, the tumor fragment is about  $2 \text{ mm}^3$ . In some

embodiments, the tumor fragment is about 3 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 4 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 5 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 6 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 7 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 8 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 9 mm<sup>3</sup>. In some embodiments, the tumor fragment is about 10 mm<sup>3</sup>. In some embodiments, the tumors are 1-4 mm × 1-4 mm × 1-4 mm. In some embodiments, the tumors are 1 mm × 1 mm × 1 mm. In some embodiments, the tumors are 2 mm × 2 mm × 2 mm. In some embodiments, the tumors are 3 mm × 3 mm × 3 mm. In some embodiments, the tumors are 4 mm × 4 mm × 4 mm.

[00301] In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are resected in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are resected in order to minimize the amount of fatty tissue on each piece.

[00302] 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% CO<sub>2</sub> and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% CO<sub>2</sub>, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% CO<sub>2</sub>. In some embodiments, at the end of the final incubation if the cell suspension contains a large number of red blood cells or dead cells, a density gradient separation using Ficoll can be performed to remove these cells.

[00303] In some embodiments, the harvested cell suspension prior to the first expansion step is called a “primary cell population” or a “freshly harvested” cell population.

[00304] In some embodiments, cells can be optionally frozen after sample harvest and stored frozen prior to entry into the expansion described in Step B, which is described in further detail below, as well as exemplified in Figure 82.

**1. Pleural effusion T-cells, ascites fluid T-cells, and TILs**

[00305] 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.

[00306] In some embodiments, the sample is an ascites fluid sample. In some embodiments, the source of the T-cells or TILs for expansion according to the processes described herein is an ascites fluid sample. In some embodiments, the sample is an ascites derived sample. In any of the foregoing embodiments, the ascites fluid sample or ascites derived sample is obtained from an endometrial cancer patient. In some embodiments, ascites fluid from the abdomen of an endometrial cancer patient may be used to obtain TILs for expansion according to the processes described herein and for treatment of the endometrial cancer patient using TILs and optional co-therapies described herein.

[00307] 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 derived from 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 manner in malignancies and such fluids in some embodiments

contain TILs. In some embodiments, wherein the disclosed methods utilize pleural fluid, the same methods may be performed with similar results using ascites or other cyst fluids containing TILs.

**[00308]** In some embodiments, the pleural or ascites fluid is in unprocessed form, directly as removed from the patient. In some embodiments, the unprocessed pleural or ascites fluid is placed in a standard blood collection tube, such as an EDTA or Heparin tube, prior to further processing steps. In some embodiments, the unprocessed pleural or ascites fluid is placed in a standard CellSave® tube (Veridex) prior to further processing steps. 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 or ascites 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.

**[00309]** In some embodiments, the pleural or ascites fluid sample from the chosen subject may be diluted. In some embodiments, the dilution is 1:10 pleural or ascites fluid to diluent. In other embodiments, the dilution is 1:9 pleural or ascites fluid to diluent. In other embodiments, the dilution is 1:8 pleural or ascites fluid to diluent. In other embodiments, the dilution is 1:5 pleural or ascites fluid to diluent. In other embodiments, the dilution is 1:2 pleural or ascites fluid to diluent. In other embodiments, the dilution is 1:1 pleural or ascites 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 or ascites 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 or ascites 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.

**[00310]** In still other embodiments, pleural or ascites fluid samples are concentrated by conventional means prior to further processing steps. In some embodiments, this pre-treatment of the pleural or ascites fluid is preferable in circumstances in which the pleural or ascites 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 or ascites fluid sample is prepared by centrifuging the pleural or ascites fluid sample after its withdrawal from the subject and resuspending the centrifugate or pellet in buffer. In some embodiments, the pleural or ascites fluid sample is subjected to multiple centrifugations and resuspensions, before it is cryopreserved for transport or later analysis and/or processing.

**[00311]** In some embodiments, pleural or ascites fluid samples are concentrated prior to further processing steps by using a filtration method. In some embodiments, the pleural or ascites fluid sample used in further processing is prepared by filtering the fluid through a filter containing a known and essentially uniform pore size that allows for passage of the pleural or ascites 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  $\mu\text{M}$ . In other embodiments the pore diameter may be 5  $\mu\text{M}$  or more, and in other embodiment, any of 6, 7, 8, 9, or 10  $\mu\text{M}$ . 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 further processing steps of the method.

**[00312]** In some embodiments, the pleural or ascites fluid sample (including, for example, the untreated pleural or ascites fluid), the diluted pleural or ascites 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 or ascites 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 or ascites 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.

**[00313]** In some embodiments, the pleural or ascites fluid sample, unprocessed, diluted or multiply centrifuged or processed as described herein above is cryopreserved at a temperature of about  $-140^{\circ}\text{C}$  prior to being further processed and/or expanded as provided herein.

#### **B. STEP B: First Expansion**

**[00314]** In some embodiments, the present methods provide for obtaining young TILs, which are capable of increased replication cycles upon administration to a subject/patient and as such may provide additional therapeutic benefits over older TILs (i.e., TILs which have further undergone more rounds of replication prior to administration to a subject/patient). Features of young TILs have been described in the literature, for example in Donia, et al., *Scand. J. Immunol.* 2012, 75, 157–167; Dudley, et al., *Clin. Cancer Res.* 2010, 16, 6122–6131; Huang, et al., *J. Immunother.* 2005, 28, 258–267; Besser, et al., *Clin. Cancer Res.* 2013, 19, OF1-OF9; Besser, et al., *J. Immunother.* 2009, 32:415–423; Robbins, et al., *J. Immunol.* 2004, 173, 7125–7130; Shen, et al., *J. Immunother.*, 2007, 30, 123–129; Zhou, et al., *J. Immunother.* 2005, 28, 53–62; and Tran, et al., *J. Immunother.*, 2008, 31, 742–751, each of which is incorporated herein by reference.

**[00315]** The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 82. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell

repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using methods referred to as process 1C, as exemplified in Figure 5 and/or Figure 6. In some embodiments, the TILs obtained in the first expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin 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 $\alpha/\beta$ ).

**[00316]** After dissection or digestion of tumor fragments, for example such as described in Step A of Figure 82, the resulting cells are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 3 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 7 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 10 to 14 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of about 11 days, resulting in a bulk TIL population, generally about  $1 \times 10^8$  bulk TIL cells.

**[00317]** In some embodiments, expansion of TILs may be performed using an initial bulk TIL expansion step (for example such as those described in Step B of Figure 82, which can include processes referred to as pre-REP) as described below and herein, followed by a second expansion (Step D, including processes referred to as rapid expansion protocol (REP) steps) as described below under Step D and herein, followed by optional cryopreservation, and followed by a second Step D (including processes referred to as restimulation REP steps)

as described below and herein. The TILs obtained from this process may be optionally characterized for phenotypic characteristics and metabolic parameters as described herein.

**[00318]** In some embodiments where TIL cultures are initiated in 24-well plates, for example, using Costar 24-well cell culture cluster, flat bottom (Corning Incorporated, Corning, NY, each well can be seeded with  $1 \times 10^6$  tumor digest cells or one tumor fragment in 2 mL of complete medium (CM) with IL-2 (6000 IU/mL; Chiron Corp., Emeryville, CA). In some embodiments, the tumor fragment is between about 1 mm<sup>3</sup> and 10 mm<sup>3</sup>.

**[00319]** In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, CM for Step B consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10 cm<sup>2</sup> gas-permeable silicon bottom (for example, G-REX10; Wilson Wolf Manufacturing, New Brighton, MN), each flask was loaded with 10–40  $\times 10^6$  viable tumor digest cells or 5–30 tumor fragments in 10–40 mL of CM with IL-2. Both the G-REX10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% CO<sub>2</sub> and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days.

**[00320]** 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.

**[00321]** 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.

[00322] 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<sup>+</sup>, Al<sup>3+</sup>, Ba<sup>2+</sup>, Cd<sup>2+</sup>, CO<sub>2</sub><sup>+</sup>, Cr<sup>3+</sup>, Ge<sup>4+</sup>, Se<sup>4+</sup>, Br, T, Mn<sup>2+</sup>, P, Si<sup>4+</sup>, V<sup>5+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, Rb<sup>+</sup>, Sn<sup>2+</sup> and Zr<sup>4+</sup>. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[00323] In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00324] 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.

[00325] In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™

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

[00326] In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-

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

[00327] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

[00328] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55μM.

[00329] 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<sup>+</sup>, Al<sup>3+</sup>, Ba<sup>2+</sup>, Cd<sup>2+</sup>, CO<sub>2</sub><sup>+</sup>, Cr<sup>3+</sup>, Ge<sup>4+</sup>, Se<sup>4+</sup>, Br, T, Mn<sup>2+</sup>, P, Si<sup>4+</sup>, V<sup>5+</sup>, Mo<sup>6+</sup>, Ni<sup>2+</sup>, Rb<sup>+</sup>, Sn<sup>2+</sup> and Zr<sup>4+</sup>. 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00330] 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.

[00331] 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 4 below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading “A Preferred Embodiment of the 1X Medium” in Table 4. 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 4 below.

TABLE 4: 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 <sub>4</sub> (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  |

[00332] In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μM), 2-mercaptoethanol (final concentration of about 100 μM).

[00333] 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.

[00334] 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 βME; also known as 2-mercaptoethanol, CAS 60-24-2).

[00335] After preparation of the tumor fragments, the resulting cells (i.e., fragments) are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum (or, in some cases, as outlined herein, in the presence of an APC cell population) with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 10 to 14 days, resulting in a bulk TIL population, generally about 1×10<sup>8</sup> bulk TIL cells. In some embodiments, the growth media during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the IL is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of 20-30×10<sup>6</sup> IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 20×10<sup>6</sup> IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 25×10<sup>6</sup> IU/mg for a 1 mg vial. In some

embodiments the IL-2 stock solution has a specific activity of  $30 \times 10^6$  IU/mg for a 1 mg vial. In some embodiments, the IL-2 stock solution has a final concentration of  $4-8 \times 10^6$  IU/mg of IL-2. In some embodiments, the IL-2 stock solution has a final concentration of  $5-7 \times 10^6$  IU/mg of IL-2. In some embodiments, the IL-2 stock solution has a final concentration of  $6 \times 10^6$  IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepared as described in Example 5. In some embodiments, the first expansion culture media comprises about 10,000 IU/mL of IL-2, about 9,000 IU/mL of IL-2, about 8,000 IU/mL of IL-2, about 7,000 IU/mL of IL-2, about 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 cell culture medium comprises about 3,000 IU/mL of IL-2. In some embodiments, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3,000 IU/mL of IL-2. In some embodiments, the 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 cell culture medium comprises between 1,000 and 2,000 IU/mL, between 2,000 and 3,000 IU/mL, between 3,000 and 4,000 IU/mL, between 4,000 and 5,000 IU/mL, between 5,000 and 6,000 IU/mL, between 6,000 and 7,000 IU/mL, between 7,000 and 8,000 IU/mL, or about 8,000 IU/mL of IL-2.

**[00336]** In some embodiments, first expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments,

the first expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In 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.

[00337] In some embodiments, first expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In 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.

[00338] In some embodiments, the cell culture medium comprises an anti-CD3 agonist antibody, e.g. 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 does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab. See, for example, Table 1.

[00339] 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.

[00340] 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.

[00341] In some embodiments, the first expansion culture medium is referred to as “CM”, an abbreviation for culture media. In some embodiments, it is referred to as CM1 (culture medium 1). In some embodiments, CM consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10cm<sup>2</sup> gas-permeable silicon bottom (for example, G-REX10; Wilson Wolf Manufacturing, New Brighton, MN), each flask was loaded with 10–40x10<sup>6</sup> viable tumor digest cells or 5–30 tumor fragments in 10–40mL of CM with IL-2. Both the G-REX10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% CO<sub>2</sub> and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days. In some embodiments, the CM is the CM1 described in the Examples, see, Example 1. In some embodiments, the first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the initial cell culture medium or the first cell culture medium comprises IL-2.

[00342] In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 82, which can include those sometimes referred to as the pre-REP) process is shortened to 3-14 days, as discussed in the examples and figures. In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 82, which can include those sometimes referred to as the

pre-REP) is shortened to 7 to 14 days, as including for example, an expansion as described in Step B of Figure 82. In some embodiments, the first expansion of Step B is shortened to 10-14 days. In some embodiments, the first expansion is shortened to 11 days, as discussed in, for example, an expansion as described in Step B of Figure 82.

**[00343]** In some embodiments, the first TIL expansion can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the first TIL expansion can proceed for 1 day to 14 days. In some embodiments, the first TIL expansion can proceed for 2 days to 14 days. In some embodiments, the first TIL expansion can proceed for 3 days to 14 days. In some embodiments, the first TIL expansion can proceed for 4 days to 14 days. In some embodiments, the first TIL expansion can proceed for 5 days to 14 days. In some embodiments, the first TIL expansion can proceed for 6 days to 14 days. In some embodiments, the first TIL expansion can proceed for 7 days to 14 days. In some embodiments, the first TIL expansion can proceed for 8 days to 14 days. In some embodiments, the first TIL expansion can proceed for 9 days to 14 days. In some embodiments, the first TIL expansion can proceed for 10 days to 14 days. In some embodiments, the first TIL expansion can proceed for 11 days to 14 days. In some embodiments, the first TIL expansion can proceed for 12 days to 14 days. In some embodiments, the first TIL expansion can proceed for 13 days to 14 days. In some embodiments, the first TIL expansion can proceed for 14 days. In some embodiments, the first TIL expansion can proceed for 1 day to 11 days. In some embodiments, the first TIL expansion can proceed for 2 days to 11 days. In some embodiments, the first TIL expansion can proceed for 3 days to 11 days. In some embodiments, the first TIL expansion can proceed for 4 days to 11 days. In some embodiments, the first TIL expansion can proceed for 5 days to 11 days. In some embodiments, the first TIL expansion can proceed for 6 days to 11 days. In some embodiments, the first TIL expansion can proceed for 7 days to 11 days. In some embodiments, the first TIL expansion can proceed for 8 days to 11 days. In some embodiments, the first TIL expansion can proceed for 9 days to 11 days. In some embodiments, the first TIL expansion can proceed for 10 days to 11 days. In some embodiments, the first TIL expansion can proceed for 11 days.

**[00344]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first

expansion, including for example during a Step B processes according to Figure 82, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 82 and as described herein.

[00345] In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 82) process is shortened to 3 to 14 days, as discussed in the examples and figures. In some embodiments, the first expansion of Step B is shortened to 7 to 14 days. In some embodiments, the first expansion of Step B is shortened to 10 to 14 days. In some embodiments, the first expansion is shortened to 11 days. In some embodiments, the first expansion, for example, Step B according to Figure 82, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

#### 1. Cytokines and Other Additives

[00346] 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.

[00347] Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is 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, or 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.

[00348] 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 other embodiments, 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. STEP C: First Expansion to Second Expansion Transition**

[00349] In some cases, the bulk TIL population obtained from the first expansion, including for example the TIL population obtained from for example, Step B as indicated in Figure 82, can be cryopreserved immediately, using the protocols discussed herein below. Alternatively, the TIL population obtained from the first expansion, referred to as the second TIL population, can be subjected to a second expansion (which can include expansions sometimes referred to as REP) and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the first TIL population (sometimes referred to as the bulk TIL population) or the second TIL population (which can in some embodiments include populations referred to as the REP TIL populations) can be subjected to genetic modifications for suitable treatments prior to expansion or after the first expansion and prior to the second expansion.

[00350] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 82) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 82) are not stored and proceed directly to the second expansion. In some embodiments, the TILs obtained from the first expansion are not cryopreserved after the first expansion and prior to the second expansion. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 7 days to 14 days from when fragmentation occurs. In some

embodiments, the transition from the first expansion to the second expansion occurs at about 14 days from when fragmentation occurs.

**[00351]** In some embodiments, the transition from the first expansion to the second expansion occurs at 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 1 day to 14 days from when fragmentation occurs. In some embodiments, the first TIL expansion can proceed for 2 days to 14 days. In some embodiments, the transition from the first expansion to the second expansion occurs 3 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 5 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 6 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 7 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 8 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 9 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 10 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 11 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 12 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 13 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 1 day to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 2 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 3 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 4 days to 11 days from when fragmentation occurs. In some



embodiments, the transition from the first expansion to the second expansion occurs 5 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 6 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 7 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 8 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 9 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 10 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs 11 days from when fragmentation occurs.

**[00352]** In some embodiments, the TILs are not stored after the first expansion and prior to the second expansion, and the TILs proceed directly to the second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 82). In some embodiments, the transition occurs in closed system, as described herein. In some embodiments, the TILs from the first expansion, the second population of TILs, proceeds directly into the second expansion with no transition period.

**[00353]** In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 82, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100 bioreactor. In some embodiments, the closed system bioreactor is a single bioreactor.

#### **D. STEP D: Second Expansion**

**[00354]** In some embodiments, the TIL cell population is expanded in number after harvest and initial bulk processing for example, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 82). This further expansion is referred to herein as the second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (REP); as well as processes as indicated in Step D of Figure 82. The second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container.

[00355] In some embodiments, the second expansion or second TIL expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 82) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the second TIL expansion can proceed for about 7 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 8 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 9 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 10 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 11 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 12 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 13 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 14 days.

[00356] In some embodiments, the second expansion can be performed in a gas permeable container using the methods of the present disclosure (including for example, expansions referred to as REP; as well as processes as indicated in Step D of Figure 82). 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  $\mu$ 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.

**[00357]** 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.

**[00358]** 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 does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

**[00359]** 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.

**[00360]** 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.

**[00361]** In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the second expansion, including for example during a Step D processes according to Figure 82, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step D processes according to Figure 82 and as described herein.

**[00362]** 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).

**[00363]** 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.

**[00364]** 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.

**[00365]** 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 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 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.

**[00366]** In some embodiments, REP and/or the second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 mL media. Media

replacement is done (generally 2/3 media replacement via respiration with fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[00367]** In some embodiments, the second expansion (which can include processes referred to as the REP process) is shortened to 7-14 days, as discussed in the examples and figures. In some embodiments, the second expansion is shortened to 11 days.

**[00368]** In some embodiments, REP and/or the second expansion may be performed using T-175 flasks and gas permeable bags as previously described (Tran, *et al.*, *J. Immunother.* **2008**, *31*, 742-51; Dudley, *et al.*, *J. Immunother.* **2003**, *26*, 332-42) or gas permeable cultureware (G-REX flasks). In some embodiments, the second expansion (including expansions referred to as rapid expansions) is performed in T-175 flasks, and about  $1 \times 10^6$  TILs suspended in 150 mL of media may be added to each T-175 flask. The TILs may be cultured in a 1 to 1 mixture of CM and AIM-V medium, supplemented with 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3. The T-175 flasks may be incubated at 37° C in 5% CO<sub>2</sub>. Half the media may be exchanged on day 5 using 50/50 medium with 3000 IU per mL of IL-2. In some embodiments, on day 7 cells from two T-175 flasks may be combined in a 3 L bag and 300 mL of AIM V with 5% human AB serum and 3000 IU per mL of IL-2 was added to the 300 mL of TIL suspension. The number of cells in each bag was counted every day or two and fresh media was added to keep the cell count between 0.5 and  $2.0 \times 10^6$  cells/mL.

**[00369]** In some embodiments, the second expansion (which can include expansions referred to as REP, as well as those referred to in Step D of Figure 82) may be performed in 500 mL capacity gas permeable flasks with 100 cm gas-permeable silicon bottoms (G-REX-100, commercially available from Wilson Wolf Manufacturing Corporation, New Brighton, MN, USA),  $5 \times 10^6$  or  $10 \times 10^6$  TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3). The G-REX-100 flasks may be incubated at 37°C in 5% CO<sub>2</sub>. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and centrifuged at 1500 rpm ( $491 \times g$ ) for 10 minutes. The TIL pellets may be re-suspended with 150 mL of fresh medium with 5% human AB serum, 3000 IU per mL of IL-2, and added back to the original G-REX-100 flasks. When TIL are expanded serially in G-REX-100 flasks, on day 7 the TIL in each G-REX-100 may be suspended in the 300 mL of media

present in each flask and the cell suspension may be divided into 3 100 mL aliquots that may be used to seed 3 G-REX-100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU per mL of IL-2 may be added to each flask. The G-REX-100 flasks may be incubated at 37° C in 5% CO<sub>2</sub> and after 4 days 150 mL of AIM-V with 3000 IU per mL of IL-2 may be added to each G-REX-100 flask. The cells may be harvested on day 14 of culture.

**[00370]** In some embodiments, the second expansion (including expansions referred to as REP) is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 mL media. In some embodiments, media replacement is done until the cells are transferred to an alternative growth chamber. In some embodiments, 2/3 of the media is replaced by respiration with fresh media. In some embodiments, alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

**[00371]** In some embodiments, the second expansion (including expansions referred to as REP) is performed and further comprises a step wherein TILs are selected for superior tumor reactivity. Any selection method known in the art may be used. For example, the methods described in U.S. Patent Application Publication No. 2016/0010058 A1, the disclosures of which are incorporated herein by reference, may be used for selection of TILs for superior tumor reactivity.

**[00372]** Optionally, a cell viability assay can be performed after the second expansion (including expansions referred to as the REP expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. In some embodiments, TIL samples can be counted and viability determined using a Cellometer K2 automated cell counter (Nexcelom Bioscience, Lawrence, MA). In some embodiments, viability is determined according to the standard Cellometer K2 Image Cytometer Automatic Cell Counter protocol.

**[00373]** In some embodiments, the second expansion (including expansions referred to as REP) of TIL can be performed using T-175 flasks and gas-permeable bags as previously described (Tran, *et al.*, **2008**, *J Immunother.*, *31*, 742–751, and Dudley, *et al.* **2003**, *J Immunother.*, *26*, 332–342) or gas-permeable G-REX flasks. In some embodiments, the second expansion is performed using flasks. In some embodiments, the second expansion is

performed using gas-permeable G-REX flasks. In some embodiments, the second expansion is performed in T-175 flasks, and about  $1 \times 10^6$  TIL are suspended in about 150 mL of media and this is added to each T-175 flask. The TIL are cultured with irradiated (50 Gy) allogeneic PBMC as “feeder” cells at a ratio of 1 to 100 and the cells were cultured in a 1 to 1 mixture of CM and AIM-V medium (50/50 medium), supplemented with 3000 IU/mL of IL-2 and 30 ng/mL of anti-CD3. The T-175 flasks are incubated at 37°C in 5% CO<sub>2</sub>. In some embodiments, half the media is changed on day 5 using 50/50 medium with 3000 IU/mL of IL-2. In some embodiments, on day 7, cells from 2 T-175 flasks are combined in a 3 L bag and 300 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to the 300 mL of TIL suspension. The number of cells in each bag can be counted every day or two and fresh media can be added to keep the cell count between about 0.5 and about  $2.0 \times 10^6$  cells/mL.

**[00374]** In some embodiments, the second expansion (including expansions referred to as REP) are performed in 500 mL capacity flasks with 100 cm<sup>2</sup> gas-permeable silicon bottoms (G-REX-100, Wilson Wolf) about  $5 \times 10^6$  or  $10 \times 10^6$  TIL are cultured with irradiated allogeneic PBMC at a ratio of 1 to 100 in 400 mL of 50/50 medium, supplemented with 3000 IU/mL of IL-2 and 30 ng/mL of anti-CD3. The G-REX-100 flasks are incubated at 37°C in 5% CO<sub>2</sub>. In some embodiments, on day 5, 250mL of supernatant is removed and placed into centrifuge bottles and centrifuged at 1500 rpm (491 g) for 10 minutes. The TIL pellets can then be resuspended with 150 mL of fresh 50/50 medium with 3000 IU/mL of IL-2 and added back to the original G-REX-100 flasks. In some embodiments where TILs are expanded serially in G-REX-100 flasks, on day 7 the TIL in each G-REX-100 are suspended in the 300 mL of media present in each flask and the cell suspension was divided into three 100 mL aliquots that are used to seed 3 G-REX-100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to each flask. The G-REX-100 flasks are incubated at 37°C in 5% CO<sub>2</sub> and after 4 days 150 mL of AIM-V with 3000 IU/mL of IL-2 is added to each G-REX-100 flask. The cells are harvested on day 14 of culture.

**[00375]** The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method



exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained in the second expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCR $\alpha/\beta$ ).

**[00376]** In some embodiments, the second expansion culture medium (*e.g.*, sometimes referred to as CM<sup>2</sup> 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.

**[00377]** 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.

**[00378]** 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00379]** 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{CO}_2^+$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ ,  $\text{Br}$ ,  $\text{T}$ ,  $\text{Mn}^{2+}$ ,  $\text{P}$ ,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

**[00380]** In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00381]** 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.

**[00382]** In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some

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

**[00383]** In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 3000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 6000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and 55mM of 2-mercaptoethanol, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific)

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

**[00384]** In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

**[00385]** In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM. In some embodiments, the final concentration of 2-mercaptoethanol in the media is 55μM.

**[00386]** 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  $\text{Ag}^+$ ,  $\text{Al}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{CO}_2$ ,  $\text{Cr}^{3+}$ ,  $\text{Ge}^{4+}$ ,  $\text{Se}^{4+}$ , Br, T,  $\text{Mn}^{2+}$ , P,  $\text{Si}^{4+}$ ,  $\text{V}^{5+}$ ,  $\text{Mo}^{6+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Rb}^+$ ,  $\text{Sn}^{2+}$  and  $\text{Zr}^{4+}$ . 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 ( $\alpha$ MEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

**[00387]** 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.

**[00388]** 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 4. 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 4. 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 4.

**[00389]** In some embodiments, the osmolality of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolality is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100  $\mu$ M), 2-mercaptoethanol (final concentration of about 100  $\mu$ M).

**[00390]** 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.

**[00391]** 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  $\beta$ ME; also known as 2-mercaptoethanol, CAS 60-24-2).

[00392] In some embodiments, the second expansion, for example, Step D according to Figure 82, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

[00393] In some embodiments, the step of rapid or second expansion is split into a plurality of steps to achieve a 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 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.

[00394] In some embodiments, the step of rapid or second expansion is split into a plurality of steps to achieve a scaling out of the culture by: (a) performing the rapid or 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.

[00395] In some embodiments, the first small scale TIL culture is apportioned into a plurality of about 2 to 5 subpopulations of TILs.

[00396] In some embodiments, the step of rapid or 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 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.

**[00397]** In some embodiments, the step of rapid or 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.

**[00398]** In some embodiments, upon the splitting of the rapid or 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.

**[00399]** 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.

**[00400]** In some embodiments, after the completion of the rapid or 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.

**[00401]** In some embodiments, the rapid or 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 or 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.



[00402] In some embodiments, the splitting of the rapid or 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.

[00403] In some embodiments, the rapid or second expansion is further performed for a period of about 7 to 11 days after the splitting. In some embodiments, the rapid or 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.

[00404] In some embodiments, the cell culture medium used for the rapid or second expansion before the splitting comprises the same components as the cell culture medium used for the rapid or second expansion after the splitting. In some embodiments, the cell culture medium used for the rapid or second expansion before the splitting comprises different components from the cell culture medium used for the rapid or second expansion after the splitting.

[00405] In some embodiments, the cell culture medium used for the rapid or 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 or 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 or second expansion before the splitting comprises IL-2, OKT-3 and APCs.

[00406] In some embodiments, the cell culture medium used for the rapid or 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 or 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 or 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 or 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.

**[00407]** In some embodiments, the cell culture medium used for the rapid or second expansion after the splitting comprises IL-2, and optionally OKT-3. In some embodiments, the cell culture medium used for the rapid or second expansion after the splitting comprises IL-2, and OKT-3. In some embodiments, the cell culture medium used for the rapid or second expansion after the splitting is generated by replacing the cell culture medium used for the rapid or 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 or second expansion after the splitting is generated by replacing the cell culture medium used for the rapid or second expansion before the splitting with fresh culture medium comprising IL-2 and OKT-3.

**[00408]** In some embodiments, the splitting of the rapid expansion occurs in a closed system.

**[00409]** In some embodiments, the scaling up of the TIL culture during the rapid or second expansion comprises adding fresh cell culture medium to the TIL culture (also referred to as feeding the TILs). In some embodiments, the feeding comprises adding fresh cell culture medium to the TIL culture frequently. In some embodiments, the feeding comprises adding fresh cell culture medium to the TIL culture at a regular interval. In some embodiments, the fresh cell culture medium is supplied to the TILs via a constant flow. In some embodiments, an automated cell expansion system such as Xuri W25 is used for the rapid expansion and feeding.

#### **1. Feeder Cells and Antigen Presenting Cells**

**[00410]** In some embodiments, the second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 82, as well as those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

[00411] 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.

[00412] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

[00413] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30 ng/mL OKT3 antibody and 3000 IU/mL IL-2.

[00414] In some embodiments, PBMCs are considered replication incompetent and accepted for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/mL OKT3 antibody and 1000-6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/mL OKT3 antibody and 2000-5000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/mL OKT3 antibody and 2000-4000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/mL OKT3 antibody and 2500-3500 IU/mL IL-2.

[00415] 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.

**[00416]** In some embodiments, the second expansion procedures described herein require a ratio of about  $2.5 \times 10^9$  feeder cells to about  $100 \times 10^6$  TIL. In other embodiments, the second expansion procedures described herein require a ratio of about  $2.5 \times 10^9$  feeder cells to about  $50 \times 10^6$  TIL. In yet other embodiments, the second expansion procedures described herein require about  $2.5 \times 10^9$  feeder cells to about  $25 \times 10^6$  TIL.

**[00417]** In some embodiments, the second expansion procedures described herein require an excess of feeder cells during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

**[00418]** 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.

**[00419]** In some embodiments, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

## **2. Cytokines and Other Additives**

**[00420]** 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.

**[00421]** Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is 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.

[00422] In some embodiments, Step D may also include the addition of OKT-3 antibody or muromonab to the culture media, as described elsewhere herein. In some embodiments, Step D may also include the addition of a 4-1BB agonist to the culture media, as described elsewhere herein. In some embodiments, Step D 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 1-alpha agonists, including proliferator-activated receptor (PPAR)-gamma agonists such as a thiazolidinedione compound, may be used in the culture media during Step D, as described in U.S. Patent Application Publication No. US 2019/0307796 A1, the disclosure of which is incorporated by reference herein.

#### **E. STEP E: Harvest TILs**

[00423] After the second expansion step, cells can be harvested. In some embodiments the TILs are harvested after one, two, three, four or more expansion steps, for example as provided in Figure 82. In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 82.

[00424] 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.

[00425] Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing systems is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term “LOVO cell processing system” also refers to any instrument or device manufactured by any vendor that can pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

[00426] In some embodiments, the harvest, for example, Step E according to Figure 82, is performed from a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the closed system bioreactor is a single bioreactor.

[00427] In some embodiments, Step E according to Figure 82, is performed according to the processes described herein. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in the Examples is employed.

[00428] In some embodiments, TILs are harvested according to the methods described in the Examples. In some embodiments, TILs between days 1 and 11 are harvested using the methods as described in the steps referred herein, such as in the day 11 TIL harvest in the Examples. In some embodiments, TILs between days 12 and 24 are harvested using the methods as described in the steps referred herein, such as in the Day 22 TIL harvest in the Examples. In some embodiments, TILs between days 12 and 22 are harvested using the methods as described in the steps referred herein, such as in the Day 22 TIL harvest in the Examples.

#### **F. STEP F: Final Formulation and Transfer to Infusion Container**

[00429] After Steps A through E as provided in an exemplary order in Figure 82 and as outlined in detailed above and herein are complete, cells 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.

[00430] In some embodiments, TILs expanded using APCs 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.

**IX. Pharmaceutical Compositions, Dosages, and Dosing Regimens**

[00411] In some embodiments, gene-edited TILs or non-gene-edited TILs made 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.

[00412] In exemplary embodiments, the gene-edited TILs provided herein include one or more cytokines selected from the group consisting of IL-12, IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof. In some embodiments, the one or more cytokines are tethered cytokines.

[00413] In some embodiments, the gene-edited TILs provided herein further comprise a nucleotide sequence that encodes an shRNA. In some embodiments, the shRNA inhibits the expression of an immune checkpoint gene. Non-limiting examples of immune checkpoint genes that may be silenced or inhibited by the shRNA include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGFβ, 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

[00414] In exemplary embodiments, the gene-edited TILs provided herein include two cytokines, wherein the first cytokine is IL-12. In some embodiments, the second cytokine is IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof.

[00415] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-15.

[00416] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-2.

[00417] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-21.

[00418] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-18.

[00419] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-6.

[00420] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-7.

[00421] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-9.

[00422] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-23.

[00423] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-27.

[00424] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-33.

[00425] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IFN gamma.



[00426] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is TNF $\alpha$ .

[00427] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IFN alpha.

[00428] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IFN beta.

[00429] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is GM-CSF.

[00430] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is GCSF.

[00431] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-15, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

[00432] In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-2, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00433]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-21, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00434]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-18, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00435]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-6, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00436]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-7, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00437]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-9, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00438]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-23, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00439]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-27, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00440]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IL-33, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00441]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IFN gamma, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00442]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is TNF $\alpha$ , and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00443]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IFN alpha, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00444]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is IFN beta, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00445]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is GM-CSF, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00446]** In exemplary embodiments, the gene-edited TILs provided herein include two membrane anchored cytokines, wherein the first cytokine is IL-12 and the second cytokine is GCSF, and an shRNA that inhibits the expression of an immune checkpoint gene selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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. In some embodiments, the gene-edited TILs provided herein further comprise a PD-1 shRNA.

**[00447]** Further provided is a therapeutic population of the gene-edited TILs or non-gene-edited TILs disclosed herein, for example, a therapeutic population of the gene-edited TILs or non-gene-edited TILs made using the method of making gene-edited TILs or non-gene-edited TILs in this disclosure. In some embodiments, the therapeutic population of the gene-edited TILs or non-gene-edited TILs may be administered to a patient in a pharmaceutical composition.

[00448] Any suitable dose of TILs can be administered. In some embodiments, from about  $2.3 \times 10^{10}$  to about  $13.7 \times 10^{10}$  TILs are administered, with an average of around  $7.8 \times 10^{10}$  TILs, particularly if the cancer is NSCLC or melanoma. In some embodiments, about  $1.2 \times 10^{10}$  to about  $4.3 \times 10^{10}$  of TILs are administered. In some embodiments, about  $3 \times 10^{10}$  to about  $12 \times 10^{10}$  TILs are administered. In some embodiments, about  $4 \times 10^{10}$  to about  $10 \times 10^{10}$  TILs are administered. In some embodiments, about  $5 \times 10^{10}$  to about  $8 \times 10^{10}$  TILs are administered. In some embodiments, about  $6 \times 10^{10}$  to about  $8 \times 10^{10}$  TILs are administered. In some embodiments, about  $7 \times 10^{10}$  to about  $8 \times 10^{10}$  TILs are administered. In some embodiments, the therapeutically effective dosage is about  $2.3 \times 10^{10}$  to about  $13.7 \times 10^{10}$ . In some embodiments, the therapeutically effective dosage is about  $7.8 \times 10^{10}$  TILs, particularly of the cancer is melanoma. In some embodiments, the therapeutically effective dosage is about  $7.8 \times 10^{10}$  TILs, particularly of the cancer is NSCLC. In some embodiments, the therapeutically effective dosage is about  $1.2 \times 10^{10}$  to about  $4.3 \times 10^{10}$  of TILs. In some embodiments, the therapeutically effective dosage is about  $3 \times 10^{10}$  to about  $12 \times 10^{10}$  TILs. In some embodiments, the therapeutically effective dosage is about  $4 \times 10^{10}$  to about  $10 \times 10^{10}$  TILs. In some embodiments, the therapeutically effective dosage is about  $5 \times 10^{10}$  to about  $8 \times 10^{10}$  TILs. In some embodiments, the therapeutically effective dosage is about  $6 \times 10^{10}$  to about  $8 \times 10^{10}$  TILs. In some embodiments, the therapeutically effective dosage is about  $7 \times 10^{10}$  to about  $8 \times 10^{10}$  TILs.

[00449] In some embodiments, the number of the TILs provided in the pharmaceutical compositions of the invention is about  $1 \times 10^6$ ,  $2 \times 10^6$ ,  $3 \times 10^6$ ,  $4 \times 10^6$ ,  $5 \times 10^6$ ,  $6 \times 10^6$ ,  $7 \times 10^6$ ,  $8 \times 10^6$ ,  $9 \times 10^6$ ,  $1 \times 10^7$ ,  $2 \times 10^7$ ,  $3 \times 10^7$ ,  $4 \times 10^7$ ,  $5 \times 10^7$ ,  $6 \times 10^7$ ,  $7 \times 10^7$ ,  $8 \times 10^7$ ,  $9 \times 10^7$ ,  $1 \times 10^8$ ,  $2 \times 10^8$ ,  $3 \times 10^8$ ,  $4 \times 10^8$ ,  $5 \times 10^8$ ,  $6 \times 10^8$ ,  $7 \times 10^8$ ,  $8 \times 10^8$ ,  $9 \times 10^8$ ,  $1 \times 10^9$ ,  $2 \times 10^9$ ,  $3 \times 10^9$ ,  $4 \times 10^9$ ,  $5 \times 10^9$ ,  $6 \times 10^9$ ,  $7 \times 10^9$ ,  $8 \times 10^9$ ,  $9 \times 10^9$ ,  $1 \times 10^{10}$ ,  $2 \times 10^{10}$ ,  $3 \times 10^{10}$ ,  $4 \times 10^{10}$ ,  $5 \times 10^{10}$ ,  $6 \times 10^{10}$ ,  $7 \times 10^{10}$ ,  $8 \times 10^{10}$ ,  $9 \times 10^{10}$ ,  $1 \times 10^{11}$ ,  $2 \times 10^{11}$ ,  $3 \times 10^{11}$ ,  $4 \times 10^{11}$ ,  $5 \times 10^{11}$ ,  $6 \times 10^{11}$ ,  $7 \times 10^{11}$ ,  $8 \times 10^{11}$ ,  $9 \times 10^{11}$ ,  $1 \times 10^{12}$ ,  $2 \times 10^{12}$ ,  $3 \times 10^{12}$ ,  $4 \times 10^{12}$ ,  $5 \times 10^{12}$ ,  $6 \times 10^{12}$ ,  $7 \times 10^{12}$ ,  $8 \times 10^{12}$ ,  $9 \times 10^{12}$ ,  $1 \times 10^{13}$ ,  $2 \times 10^{13}$ ,  $3 \times 10^{13}$ ,  $4 \times 10^{13}$ ,  $5 \times 10^{13}$ ,  $6 \times 10^{13}$ ,  $7 \times 10^{13}$ ,  $8 \times 10^{13}$ , and  $9 \times 10^{13}$ . In some embodiments, the number of the TILs provided in the pharmaceutical compositions of the invention is in the range of  $1 \times 10^6$  to  $5 \times 10^6$ ,  $5 \times 10^6$  to  $1 \times 10^7$ ,  $1 \times 10^7$  to  $5 \times 10^7$ ,  $5 \times 10^7$  to  $1 \times 10^8$ ,  $1 \times 10^8$  to  $5 \times 10^8$ ,  $5 \times 10^8$  to  $1 \times 10^9$ ,  $1 \times 10^9$  to  $5 \times 10^9$ ,  $5 \times 10^9$  to  $1 \times 10^{10}$ ,  $1 \times 10^{10}$  to  $5 \times 10^{10}$ ,  $5 \times 10^{10}$  to  $1 \times 10^{11}$ ,  $5 \times 10^{11}$  to  $1 \times 10^{12}$ ,  $1 \times 10^{12}$  to  $5 \times 10^{12}$ , and  $5 \times 10^{12}$  to  $1 \times 10^{13}$ .

[00450] 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.

[00451] 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.

[00452] 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.

[00453] 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.

**[00454]** 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.

**[00455]** 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.

**[00456]** 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.

**[00457]** 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.

**[00458]** In some embodiments, an effective dosage of TILs is about  $1 \times 10^6$ ,  $2 \times 10^6$ ,  $3 \times 10^6$ ,  $4 \times 10^6$ ,  $5 \times 10^6$ ,  $6 \times 10^6$ ,  $7 \times 10^6$ ,  $8 \times 10^6$ ,  $9 \times 10^6$ ,  $1 \times 10^7$ ,  $2 \times 10^7$ ,  $3 \times 10^7$ ,  $4 \times 10^7$ ,  $5 \times 10^7$ ,  $6 \times 10^7$ ,  $7 \times 10^7$ ,  $8 \times 10^7$ ,  $9 \times 10^7$ ,  $1 \times 10^8$ ,  $2 \times 10^8$ ,  $3 \times 10^8$ ,  $4 \times 10^8$ ,  $5 \times 10^8$ ,  $6 \times 10^8$ ,  $7 \times 10^8$ ,  $8 \times 10^8$ ,  $9 \times 10^8$ ,  $1 \times 10^9$ ,  $2 \times 10^9$ ,  $3 \times 10^9$ ,  $4 \times 10^9$ ,  $5 \times 10^9$ ,  $6 \times 10^9$ ,  $7 \times 10^9$ ,  $8 \times 10^9$ ,  $9 \times 10^9$ ,  $1 \times 10^{10}$ ,  $2 \times 10^{10}$ ,  $3 \times 10^{10}$ ,  $4 \times 10^{10}$ ,  $5 \times 10^{10}$ ,  $6 \times 10^{10}$ ,  $7 \times 10^{10}$ ,  $8 \times 10^{10}$ ,  $9 \times 10^{10}$ ,  $1 \times 10^{11}$ ,  $2 \times 10^{11}$ ,  $3 \times 10^{11}$ ,  $4 \times 10^{11}$ ,  $5 \times 10^{11}$ ,  $6 \times 10^{11}$ ,  $7 \times 10^{11}$ ,  $8 \times 10^{11}$ ,  $9 \times 10^{11}$ ,  $1 \times 10^{12}$ ,  $2 \times 10^{12}$ ,  $3 \times 10^{12}$ ,  $4 \times 10^{12}$ ,  $5 \times 10^{12}$ ,  $6 \times 10^{12}$ ,  $7 \times 10^{12}$ ,  $8 \times 10^{12}$ ,  $9 \times 10^{12}$ ,  $1 \times 10^{13}$ ,  $2 \times 10^{13}$ ,  $3 \times 10^{13}$ ,  $4 \times 10^{13}$ ,  $5 \times 10^{13}$ ,  $6 \times 10^{13}$ ,  $7 \times 10^{13}$ ,  $8 \times 10^{13}$ , and  $9 \times 10^{13}$ . In some embodiments, an effective dosage of TILs is in the range of  $1 \times 10^6$  to  $5 \times 10^6$ ,  $5 \times 10^6$  to  $1 \times 10^7$ ,  $1 \times 10^7$  to  $5 \times 10^7$ ,  $5 \times 10^7$  to  $1 \times 10^8$ ,  $1 \times 10^8$  to  $5 \times 10^8$ ,  $5 \times 10^8$  to  $1 \times 10^9$ ,  $1 \times 10^9$  to  $5 \times 10^9$ ,  $5 \times 10^9$  to  $1 \times 10^{10}$ ,  $1 \times 10^{10}$  to  $5 \times 10^{10}$ ,  $5 \times 10^{10}$  to  $1 \times 10^{11}$ ,  $5 \times 10^{11}$  to  $1 \times 10^{12}$ ,  $1 \times 10^{12}$  to  $5 \times 10^{12}$ , and  $5 \times 10^{12}$  to  $1 \times 10^{13}$ .

**[00459]** 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 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.

**[00460]** 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.

[00461] 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.

[00462] In other embodiments, the invention provides an infusion bag comprising the therapeutic population of TILs described in any of the preceding paragraphs above.

[00463] 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.

[00464] In other embodiments, the invention provides an infusion bag comprising the TIL composition described in any of the preceding paragraphs above.

[00465] In other embodiments, the invention provides a cryopreserved preparation of the therapeutic population of TILs described in any of the preceding paragraphs above.

[00466] 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.

[00467] 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.

[00468] 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.

[00469] In other embodiments, the invention provides a cryopreserved preparation of the TIL composition described in any of the preceding paragraphs above.

## **X. Methods of Treating Cancer Patients**

[00470] In some embodiments, a therapeutic population of non-gene-edited TILs

made using the methods of the present disclosure are administered to a patient for the treatment of cancer. In some embodiments, provided herein is a method of treating a cancer in a patient in need thereof comprising:

- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
- b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
- c) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, and L-arginine; and
- d) administering the therapeutic population of gene-edited TILs to the patient.

**[00471]** In some embodiments, provided herein is a method of treating a cancer in a patient in need thereof comprising:

- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
- b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
- c) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, L-arginine, and NAD<sup>+</sup>; and
- d) administering the therapeutic population of gene-edited TILs to the patient.

**[00472]** In some embodiments, gene-edited TILs using the methods of the present disclosure are administered to a patient for the treatment of cancer. In some embodiments, provided herein is a method of treating a cancer in a patient in need thereof comprising:

- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
- b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
- c) performing a second expansion by culturing the population of TILs in a second cell culture medium;

- d) at any time, transducing the population of TILs with the recombinant lentiviral particle disclosed herein to produce a therapeutic population of gene-edited TILs; and
- e) administering the therapeutic population of gene-edited TILs to the patient.

[00473] In some embodiments, the method further comprises activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle. In some embodiments, the activating step comprises contacting the population of TILs with a cytokine selected from the group consisting of IL-2, IL-15, IL-21, IL-7, and a combination thereof. In some embodiments, the activating step comprises contacting the population of TILs with TransAct. In some embodiments, the transducing step is conducted in the presence of RetroNectin or Vectofusin-1. In some embodiments, the transducing step comprises centrifugation. In some embodiments, the transducing step is conducted in the presence of Lentibooster. In some embodiments, the transducing step is conducted after the first expansion step and before the second expansion step. In some embodiments, the activating step is conducted after the first expansion step and before the second expansion step. In some embodiments, the method further comprises resting the population of TILs for 1 day or 2 days after the transducing step.

[00474] In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days. In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day. In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient. In some embodiments, the method further comprises a step of treating the patient with an IL-2 regimen starting on the same day as the administration of TILs to the patient. In some embodiments, the IL-2 regimen is a high-dose

IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance. In some embodiments, the method does not comprise a step of treating the patient with an IL-2 regimen. In some embodiments, the therapeutic population of TILs comprises from about  $2.3 \times 10^{10}$  to about  $13.7 \times 10^{10}$  TILs.

**[00475]** In some embodiments, the cancer 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) and small-cell lung cancer), melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris 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.

**[00476]** 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.

[00477] 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.

[00478] In some embodiments, the cancer is metastatic melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma). In some embodiments, the cancer is lung cancer (including non-small-cell lung cancer (NSCLC) and small-cell lung cancer). In some embodiments, the cancer is cervical cancer. In some embodiments, the cancer is head and neck squamous cell carcinoma (HNSCC). In some embodiments, the cancer is endometrial cancer. In some embodiments, the cancer is ovarian cancer.

#### 1. Lymphodepletion Preconditioning of Patients

[00479] 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/m<sup>2</sup>/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.

[00480] In some embodiments, the patient receives a reduced intensity non-myeloablative lymphodepletion regimen. In some embodiments, the reduced intensity non-myeloablative lymphodepletion regimen comprises administration of cyclophosphamide at a dose of about 250-750 mg/m<sup>2</sup>/day. In some embodiments, cyclophosphamide is administered at a dose of about 250 mg/m<sup>2</sup>/day. In some embodiments, cyclophosphamide is administered at a dose of about 500 mg/m<sup>2</sup>/day. In some embodiments, cyclophosphamide is administered

at a dose of about 750 mg/m<sup>2</sup>/day. In some embodiments, cyclophosphamide is administered for three or four days. In some embodiments, the administration of cyclophosphamide is followed by administration of fludarabine at a dose of 30 mg/m<sup>2</sup>/day. In some embodiments, fludarabine is administered for three, four, or five days. In some embodiments, the cyclophosphamide is administered with mesna. In some embodiments, the patient receives no non-myeloablative lymphodepletion regimen.

**[00481]** 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.

**[00482]** 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.

**[00483]** 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.

**[00484]** 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/m<sup>2</sup>/day, 150 mg/m<sup>2</sup>/day, 175 mg/m<sup>2</sup>/day, 200 mg/m<sup>2</sup>/day, 225 mg/m<sup>2</sup>/day, 250 mg/m<sup>2</sup>/day, 275 mg/m<sup>2</sup>/day, or 300 mg/m<sup>2</sup>/day. In some embodiments, the cyclophosphamide is administered intravenously (*i.e.*, i.v.) In some embodiments, the cyclophosphamide treatment is administered for 2-7 days at 35 mg/kg/day. In some embodiments, the cyclophosphamide treatment is administered for 4-5 days at 250 mg/m<sup>2</sup>/day i.v. In some embodiments, the cyclophosphamide treatment is administered for 4 days at 250 mg/m<sup>2</sup>/day i.v.

**[00485]** In some embodiments, lymphodepletion is performed by administering the fludarabine and the cyclophosphamide together to a patient. In some embodiments, fludarabine is administered at 25 mg/m<sup>2</sup>/day i.v. and cyclophosphamide is administered at 250 mg/m<sup>2</sup>/day i.v. over 4 days.

**[00486]** In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for five days.

**[00487]** In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days and administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/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.

**[00488]** In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 50 mg/m<sup>2</sup>/day for two days and administration of fludarabine at a dose of about 25 mg/m<sup>2</sup>/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.

**[00489]** In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 50 mg/m<sup>2</sup>/day for two days and administration of fludarabine at a dose of about 20 mg/m<sup>2</sup>/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.

[00490] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 40 mg/m<sup>2</sup>/day for two days and administration of fludarabine at a dose of about 20 mg/m<sup>2</sup>/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.

[00491] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of about 40 mg/m<sup>2</sup>/day for two days and administration of fludarabine at a dose of about 15 mg/m<sup>2</sup>/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.

[00492] In some embodiments, the lymphodepletion is performed by administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.

[00493] 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.

[00494] 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.

[00495] 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.

[00496] 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/m<sup>2</sup> intravenous fludarabine. In some embodiments, the regimen further comprises 25 mg/m<sup>2</sup> intravenous fludarabine on days -5 and -1 (*i.e.*, days 0 through 4). In some embodiments, the regimen further comprises 25 mg/m<sup>2</sup> intravenous fludarabine on days -5 and -1 (*i.e.*, days 0 through 4).

[00497] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for five days.

[00498] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for five days.

[00499] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days

[00500] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.

[00501] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day.

[00502] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.

[00503] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.

[00504] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 11.

TABLE 11. 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 <sup>2</sup> /day | X  | X  | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    |    |    | X |   |   |   |   |

[00505] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 12.

TABLE 12. 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 <sup>2</sup> /day | X  | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    |    | X |   |   |   |   |

[00506] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 13.

TABLE 13. 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 <sup>2</sup> /day | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    | X |   |   |   |   |

[00507] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 14.

TABLE 14. 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 <sup>2</sup> /day |    |    | X  | X  | X  |   |   |   |   |   |

| Day          | -5 | -4 | -3 | -2 | -1 | 0 | 1 | 2 | 3 | 4 |
|--------------|----|----|----|----|----|---|---|---|---|---|
| TIL infusion |    |    |    |    |    | X |   |   |   |   |

[00508] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 15.

TABLE 15. 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 <sup>2</sup> /day | X  | X  | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    |    |    | X |   |   |   |   |

[00509] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 16.

TABLE 16. 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 <sup>2</sup> /day | X  | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    |    | X |   |   |   |   |

[00510] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 17.

TABLE 17. 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 <sup>2</sup> /day | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    | X |   |   |   |   |

[00511] In some embodiments, the non-myeloablative lymphodepletion regimen is administered according to Table 18.

TABLE 18. 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 <sup>2</sup> /day |    |    | X  | X  | X  |   |   |   |   |   |
| TIL infusion                          |    |    |    |    |    | X |   |   |   |   |

[00512] 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.

[00513] In some embodiments, the non-myeloablative lymphodepletion regimen comprises melphalan administered to a total dose of 100 mg/m<sup>2</sup> over the course of 1, 2, or 3 days prior to the day of TIL infusion. In some embodiments, the non-myeloablative lymphodepletion regimen comprises melphalan administered to a total dose of 200 mg/m<sup>2</sup> over the course of 1, 2, or 3 days prior to the day of TIL infusion. In some embodiments, the non-myeloablative lymphodepletion regimen comprises melphalan administered to a total dose of 100 mg/m<sup>2</sup> and fludarabine administered at a dose of 30 mg/m<sup>2</sup>/day over the course of 1, 2, or 3 days prior to the day of TIL infusion. In some embodiments, the non-myeloablative lymphodepletion regimen comprises melphalan administered to a total dose of 200 mg/m<sup>2</sup> and fludarabine administered at a dose of 30 mg/m<sup>2</sup>/day over the course of 1, 2, or 3 days prior to the day of TIL infusion.

[00514] In some embodiments, the non-myeloablative lymphodepletion regimen comprises administration of an anti-CD45 antibody. In some embodiments, the non-myeloablative lymphodepletion regimen comprises administration of an anti-CD45 antibody-drug conjugate. In some embodiments, the non-myeloablative lymphodepletion regimen comprises administration of an anti-CD45 antibody-radioisotope conjugate. In some embodiments, the non-myeloablative lymphodepletion regimen comprises administration of apamistamab-<sup>131</sup>I. In some embodiments, the non-myeloablative lymphodepletion regimen comprises apamistamab-<sup>131</sup>I administered at a dose of 25 mCi, 50 mCi, 75 mCi, 100 mCi, 150 mCi, or 200 mCi between 2 and 9 days prior to TIL infusion. In some embodiments, the non-myeloablative lymphodepletion regimen comprises apamistamab-<sup>131</sup>I administered at a dose of 25 mCi to 200 mCi between 2 and 9 days prior to TIL infusion. In some

embodiments, the non-myeloablative lymphodepletion regimen comprises apamistamab-<sup>131</sup>I administered at a dose of 50 mCi to 150 mCi between 4 and 8 days prior to TIL infusion. In some embodiments, the non-myeloablative lymphodepletion regimen comprises apamistamab-<sup>131</sup>I administered at a dose of about 75 mCi about 6 days prior to TIL infusion. In some embodiments, the non-myeloablative lymphodepletion regimen comprises apamistamab-<sup>131</sup>I administered at a dose of about 100 mCi about 7 days prior to TIL infusion.

**[00515]** In some embodiments, the TIL infusion used with the foregoing embodiments of myeloablative lymphodepletion regimens may be any TIL composition described herein, including TIL products genetically modified to include one or more immunomodulatory agents attached to its surface, and may also include infusions of MILs and PBLs in place of the TIL infusion, as well as the addition of alternative lymphodepletion regimens including the anti-CD52 antibody alemtuzumab, or variants, fragments, antibody-drug conjugates, or biosimilars thereof.

## 2. IL-2 Regimens

**[00516]** 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.

**[00517]** 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  $18 \times 10^6$  IU/m<sup>2</sup> aldesleukin, or a biosimilar or variant thereof, administered intravenously over 6 hours, followed by  $18 \times 10^6$  IU/m<sup>2</sup> administered intravenously over 12 hours, followed by  $18 \times 10^6$  IU/m<sup>2</sup> administered intravenously over 24 hours, followed by  $4.5 \times 10^6$  IU/m<sup>2</sup> 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/m<sup>2</sup> on day 1, 9,000,000 IU/m<sup>2</sup> on day 2, and 4,500,000 IU/m<sup>2</sup> on days 3 and 4.

**[00518]** In some embodiments, a reduced-dose IL-2 regimen comprises a reduce number, e.g., 1, 2, 3, 4, or 5, doses of 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance. In some embodiments, the patient receives no IL-2 regimen.

**[00519]** 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; Hartemann, *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  $18 \times 10^6$  IU per m<sup>2</sup> 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  $18 \times 10^6$  IU per m<sup>2</sup> per 24 hours, optionally followed by 3 weeks without IL-2 therapy, after which additional cycles may be administered.

**[00520]** 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 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.



**[00521]** 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.

**[00522]** 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.

**[00523]** 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.

**[00524]** In some embodiments, the IL-2 regimen comprises administration of an IL-2 fragment grafted onto an antibody backbone. In some embodiments, the IL-2 regimen comprises administration of an antibody-cytokine grafted protein that binds the IL-2 low affinity receptor. In some embodiments, the antibody cytokine grafted 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 grafted into a CDR of the  $V_H$  or the  $V_L$ , wherein the antibody cytokine grafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the antibody cytokine grafted 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 grafted into a CDR of the  $V_H$  or the  $V_L$ , wherein the IL-2 molecule is a mutein, and wherein the antibody cytokine grafted 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.

[00525] 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.

[00526] 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.

[00527]

EXAMPLES

[00528] 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

[00529] 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.

[00530] 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 19 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 19. Preparation of CM1

| Ingredient                           | Final concentration | Final Volume 500 mL | Final Volume 1L |
|--------------------------------------|---------------------|---------------------|-----------------|
| RPMI1640                             | NA                  | 450 mL              | 900 mL          |
| Human AB serum, heat-inactivated 10% | 50 mL               | 100 mL              |                 |
| 200mM L-glutamine                    | 2 mM                | 5 mL                | 10 mL           |

|                            |               |        |      |
|----------------------------|---------------|--------|------|
| 55mM BME                   | 55 $\mu$ M    | 0.5 mL | 1 mL |
| 50mg/mL gentamicin sulfate | 50 $\mu$ g/mL | 0.5 mL | 1 mL |

[00531] On the day of use, prewarmed required amount of CM1 in 37°C water bath and add 6000 IU/mL IL-2.

[00532] Additional supplementation may be performed as needed according to Table 20.

TABLE 20. 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<br>10,000 $\mu$ g/mL streptomycin | 1:100    | 100 U/mL penicillin<br>100 $\mu$ g/mL streptomycin |
| Amphotericin B          | 250 $\mu$ g/mL                                           | 1:100    | 2.5 $\mu$ g/mL                                     |

### **Preparation of CM2**

[00533] 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**

[00534] 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**

[00535] 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: TRANSDUCTION OF TILS USING LENTIVIRAL PARTICLES**

[00536] TIL Activation

[00537] Pre-REP'ed TILs were suspended at 1e6/mL CM2 without gentamicin + 300IU/mL IL-2 in a 24 well plate at 2mL per well. Incubated overnight.

[00538] A 20x cocktail of IL-15, IL-7 was prepared for the addition of 100uL per well.

- Final concentrations: IL-15 (10ng/mL), IL-7 (20ng/mL) in CM2 without gentamicin
- Example: 10 wells needed
  - 10 wells x 100uL cocktail = 1mL
    - Add IL-15 to a concentration of 200ng/mL
    - Add IL-7 to a concentration of 400ng/mL
- Note: Remove 120uL from each well before adding TransACT, IL-15, and IL-7 so that the final volume is 2mL

[00539] 100uL of 20x cytokine cocktail was added to each TIL culture within the 24 well plate. Then, 20uL TransACT was added to each well. Each well was pipette mixed gently to evenly disperse TILs and to mix with stimulation cocktail. Incubated for 2 days.

[00540] Gene Transduction and Resting

[00541] A non-tissue culture 48 well plate with Retronectin (diluted 1:100 in PBS) was coated by adding 250uL per well. Wrapped plate in parafilm and then incubated at 4°C overnight.

[00542] The coating solution was removed from retronectin plate and replace with 250uL 2% BSA Fraction V. Note: The 2% BSA Fraction V was added immediately so that the well did not dry out.

[00543] Incubated at RT for 30min for blocking.

[00544] Blocking solution was removed. The calculated volumes of viral supernatant were added and wrapped in parafilm. Centrifuged for 2000xg, 90min, 32 °C. Note: centrifuge was warmed prior to adding plate.

[00545] Incubate at RT for 30min for blocking. Remove blocking solution. The following was added:

1. 100uL of TIL suspension (1e5 cells)
2. 300uL CM2 + IL-15 (20ng/mL) + IL-7 (40ng/mL) + Lentibooster 1:50
3. Lentivirus (produced using the lentiviral particle production protocol in Example 3) with MOI in a range of 10-40
4. Adjust with CM2 w/o gentamicin, total volume 600uL per well

[00546] Centrifuged 2000xg, 45min, 32 °C. Placed in incubator and incubated for 3 days.

### EXAMPLE 3: LENTIVIRAL PARTICLE PRODUCTION PROTOCOL

[00547] Resuspended 293T cells in virus culture medium, 6E6/10ml HEK293T cells in 10CM dish. Virus making culture medium: 10%FBS, DMEM (high glucose, Glutamine) sodium pyruvate (1% W/v), Sodium Bicarbonate (0.075%) or HEPES no Pen/strep.

[00548] On the next day, a Gag/Pol helper plasmid, a Rev helper plasmid, a BaEVTR or VSV-G helper plasmid, and a transfer vector were mixed at 1 µg/µL concentration at a 1:1 molar ratio. Following mixing, 30 µL of *TransIT*<sup>®</sup>-Lenti reagent (Mirus Bio) was added and incubated at room temperature for 10 min in 1mL Opti-MEM<sup>™</sup> media per HEK 293T dish.

[00549] HEK293T cells were then incubated at 37°C, 5% CO<sub>2</sub> for 2 days.

[00550] Viral particle harvesting: Culture supernatant was removed from dishes and fresh media was added to each dish, centrifuged at 200 g for 5 min to remove cellular debris, and then filtered using a .45 µM filter. Viral supernatant was then concentrated via ultracentrifugation for 2 hrs at 60,000 g. Following ultracentrifugation, supernatant was removed and viral pellet was redissolved in Opti-MEM<sup>™</sup> media.

[00551] The viral particle harvesting step may be repeated on the following day to optimize viral production.

**EXAMPLE 4: PRODUCTION AND CHARACTERIZATION OF MODIFIED TILs WITH MEMBRANE ANCHORED IL-15 AND IL-21 IMMUNOMODULATORY FUSION PROTEINS**

**[00552] Materials and Methods**

**[00553] Virus preparation and T cell transduction**

**[00554]** To make lentivirus, pLenti-vectors containing tethered cytokine gene sequence (Table 21) and packaging helper vectors (VSV-G, Gag/Pol) were con-transfected into 293T cells. Lentivirus supernatant was collected from day 2-3 culture supernatants followed by ultracentrifuge (120,000 g) to concentrate lentivirus for TIL transduction. Before T cell transduction, pre-REP cells were stimulated with TransACT (1:100) for 2 days. 1E5 activated pre-REP cells were added into Retronectin pre-coated 48 well plates with concentrated lentivirus. Two days after gene transduction, pre-REP cells were harvested for REP process or further phenotypic characterization and functional assays.

TABLE 21. Membrane anchored IL-15 and IL-21 fusion protein DNA sequences

| Construct | DNA sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| teIL-15   | atggactggacttgatactcttctggtcgctgccgcccacacgggtgactctaattgggtcaacgtgatcagcactgaagaagatcgaggactgatccagagcatgcacatcgacgccacactgtacaccagtgccg atgtgcaccctagctgcaaaagtaccgccatgaagtgttctgctggaactgcaagtatcagcctggaa agcggcgacgccagatccacgataccgtggaaaatctgatcatcctggccaacaacagcctgtccagc aacggcaatgtgaccgagagcggtgcaaaagagtgcgaggaactggaagaaaagaacatcaaaagatt tctgcagagcttcgtccacatcgtgcagatgttcatcaacacctatcagggtggcgggtggaagcggaggtg gcggtagtggcggcgaggctcaggcgcgagggttcggcgaggatctctcagctcctgccatctt gggclatcaccctgattagtgtgaatgggatcttgcacatctgttctcactactgttctcctcccggtgca gagagagaaggcgcaacgaaagactgcggagagaaagcgtcagaccctg (SEQ ID NO:153)                                                                                                                              |
| teIL-21   | atggattggacctggattctgttcctggtggccgctgccacaagagtgcatagccaggggccaagaccggc acatgatccggatgagacagctgatcagatcgtggaccagctgaagaactacgtgaacacctgtgtgcc tgagttctgctgctcctgaggacgtggaaacaaattcgagtggtccgcttcagctgctccagaagg ccagctgaaaagcgccaacaccggcaacaacgagcggatcatcaacgtgtccatcaagaagctgaag cgggaagcctcctagcaccaatgccggaagaaggcagaagcacagactgacctgtcctagctgcgacag ctacgagaagaagcctccaaaagagttcctggaacgggttaagagcctgctgcagaagatgtaccaccag cacctgagcagcagaacccacggctctgaagattctagcggaggcggagggaagtgtggtggcgaggttct ggtggcgggtgatcaggcgggtggcgatctggcgcgaggcagtggtggaggtggaagcgggtgtg gtggtcttgccggaggcggtagcggcgaggcgatcttcaattgctgcctagctgggccatcacact gatctccgtgaacggcatcttctgtatctgctgacctactgctcgccttagatgcagagagcggag aagaacgagcggctgagaagagaatctgtgcggcctgtt (SEQ ID NO:154) |

|              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| teIL-15/IL21 | atggattggacctggattctgttctggtggccgctgccacaagagtgcatagccaggccaagaccggc<br>acatgatccggatgagacagctgacgacatcgtggaccagctgaagaactacgtgaacgacctggtgcc<br>tgagttcctgctcctgagggacgtggaacaaattgcgagtgtccgcttcagctgctccagaagg<br>cccagctgaaaagcgccaacaccggcaacaacgagcggatcatcaacgtgtccatcaagaagctgaag<br>cggaagcctctagcaccaatccggaagaaggcagaagcacagactgacctgtcctagctgcgacag<br>ctacgagaagaagcctccaaaagagttcctggaacggttcaagagcctgctgcagaagatgatccaccag<br>cacctgagcagcagaacccacggctctgaagattctagcggaggcggagggaagtgtggcggaggttct<br>ggtggcgggtggtatcaggcgggtggcggatctggcggcggaggcagtgccggaggtggaagcgggtgtg<br>gtggctctggcggaggcggtagcggcggaggcggatcttcaattgctgcctagctgggccatcacact<br>gatctcctggaacggcatctctgtatctgctgacctactgtctcggcccctagatgcagagagcggag<br>aagaacgagcggctgagaagagaatctgtcggcctgttagagccaagatctggaagcggcgcca<br>ccaactttagcctgctgaacaggctggcgacgtggaagagaacctggacctggaactggatggat<br>actcttctggtcgtgccgcccacacgggtgcacttaattgggtcaacgtgatcagcgacctgaagaagat<br>cgaggacctgatccagagcatgcacatcgacccacactgtacaccgagtcagatgtcacccctagctgc<br>aaagtgaccgcatgaagtgtttctgtggaactgcaagtgtcagcctggaagcggcgacgccagca<br>tccacgataccgtggaatctgatcctggccaacaacagcctgtccagcaacggcgaatgtgaccga<br>gagcggctgcaaaaggtgcgaggaaactggaagaaaagaacatcaagagtttctgcagagcttcgtcca<br>catcgtgcagatgttcatcaacacctcatcagggtggcgggtggaagcggaggtggcggtagtggcggcgg<br>aggctcaggcggcggagggtccggcggaggatctctcagctcctgccatctgggctatcacctgatla<br>gtgtgaatgggatcttgtcatctgtgtctcacgtactgttctgctccccgggtgcagagagagaaggcgcaa<br>cgaaagactgcggagagaaagcgtcagaccgtg (SEQ ID NO:155) |
|--------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

[00555] Flow cytometry

[00556] To check mIL-15 expression in transduced cells, biotin-conjugate IL-15 (biolgend, San Diego, CA) plus streptavidin-BV421 (Biolegend, San Diego, CA) were used to stain transduced cells. To check mIL-21 expression, PE-conjugated IL-21 antibodies were used to stain transduced cells. To check T cell division, CellTrace™ Violet Cell Proliferation dye (ThermoFisher scientific, Waltham, MA) was used to pre-label T cells. T cell division was analyzed after 5 days in ex vivo culture in presence or absence of IL-2 (20IU/ml). To check T cell activation and differentiation, antibodies from BD Bioscience, Biolegend, Thermofisher were used for phenotypic characterization. Data was acquired with a BioRad ZE5 flow cytometer and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR).

[00557] T cell count and viability

[00558] REP cells were harvested after TIL expansion. Live cell numbers were assessed by AOPI assay using Cellometer K2 cell counter machine (Nexcelom, Lawrence, MA).

[00559] Pre-REP and REP process

[00560] Human tumor samples were dissected into ~3 mm pieces and cultured in Grex10 with recommended culture medium containing IL-2 (6000IU/ml). After 11 days of culture, pre-REP cells were harvested for 2-day activation and 2-day lentiviral transduction. Transduced Pre-REP cells were then propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and 3,000 IU/ml of IL-2 for another 11 days.

[00561] **Results**

[00562] Expression of mIL-15/mIL-21 after gene transduction

[00563] After gene transduction, transduced pre-REP cells were ex vivo expanded with 300IU/ml IL-2 for another 5 days. By flow cytometry staining, transduced pre-REP cells exhibited expression of mIL-15 and mIL-21. As shown in Figure 1A, there was a 54.8% expression of mIL-15 in mIL-15 lentiviral transduced TILs, a 53.5% expression of mIL-21 in mIL-21 transduced TILs and a 36.5% expression of mIL-15/mIL-21 double positive cells in mIL-15/IL21 transduced TILs. Un-transduced cells were used as negative control.

[00564] mIL-15 pre-REP cells exhibit sustained activation of pSTAT5 signaling and showed increased proliferation

[00565] After verification of surface cytokine expression, T cell functionality was examined. Phosphorylation of STAT5 is a hallmark of T cell activation upon gamma chain cytokine stimulation. In conditions of serum starvation, mIL-15, mIL-21, and mIL-15/IL-21 lentiviral transduced cells displayed sustained pSTAT5 signaling indicating that mIL-15 is functional. As shown in Figure 1B, mIL-15 is superior to mIL-21 in inducing pSTAT5 activation. Further, cell proliferation of mIL-15 lentiviral transduced TILs was examined by CellTrace proliferation assay. CellTrace violet pre-labeled TILs were ex vivo cultured for 5 days in the presence or absence of IL-2. As shown in Figure 1C, mIL-15 TILs exhibit superior proliferative capacity in comparison with mIL-21 or mIL-15/IL-21 TILs in the presence or absence of IL-2. mIL-15 TILs can proliferate in the absence of IL-2, indicating mIL-15 can substitute for IL-2 to provide a proliferative signal.

[00566] Expression of mIL-15/mIL-21 after REP process

[00567] After lentiviral transduction, TILs were expanded for 11 days in a REP process. Surface expression of membrane-bound cytokine receptors were then assessed by flow cytometry. As shown in Figure 2A, there was a 31.1% expression of mIL-15 in mIL-15 TILs, a 63% expression of mIL-21 in mIL-21 TILs and a 10.7% expression of mIL-15/mIL-



21 double positive cells in mIL-15/IL21 TILs. Un-transduced cells were used as a negative control.

**[00568]**      Expression of mIL-15/mIL-21 promote CD8 T cell expansion in REP process

**[00569]**      In harvested REP cells, an increased percentage of CD8+ T cells was detected in mIL-15, mIL-21 and mIL-15/IL-21 lentiviral transduced TILs (Figures 2B and 2C). This is consistent with previous reports showing that IL-15 and IL-21 act as a potent stimulator, which preferentially stimulates the proliferation and survival of CD8 T cells.

**[00570]**      Phenotype of mIL-15/mIL-21 expressing REP TIL

**[00571]**      To characterize the immune regulatory effect of mIL-15 and mIL-21 on REP cells, phenotypic analysis was performed with freshly thawed REP cells in both gated CD8+CD3+T cells (Figure 3) and CD4+CD3+ T cell subset (Figure 4). We found mbIL-15 significantly downregulate CD25 (IL-2Ralpha) expression. As IL-2, IL-15 are in competition in using IL-2R beta and IL-2R gamma, suggesting a negative feedback mechanism in regulating CD25. Also, mIL-15 appears to activate T cells, characterized with higher expression of TIM3, TOX, while IL-21 exhibit antagonist effect. Further, both mIL-15 and mIL-21 downregulate expression of Eomes. No large difference was observed in the expression of other surface marker such as PD-1, CD27, CXCR3, etc.

#### **EXAMPLE 5: PRODUCTION AND CHARACTERIZATION OF MODIFIED TILs WITH MEMBRANE ANCHORED IL-15 AND IL-21 IMMUNOMODULATORY FUSION PROTEINS**

**[00572]**      For this study, modified TILs with membrane anchored IL-15 and/or IL-21 immunomodulatory fusion proteins will be made, wherein expression of the membrane anchored immunomodulatory IL-15 and IL-21 is controlled using a NFAT promoter that includes NFAT responsive elements linked to a minimal human IL-2 promoter, see, e.g., Table 23. The modified TILs will be characterized as set forth below.

**[00573]**      **Materials and Methods**

**[00574]**      Virus preparation and T cell transduction

**[00575]**      To make lentivirus, pLenti-vectors containing the tethered cytokine gene sequence (Table 22) and packaging helper vectors (BaEV-TR, Gag/Pol) will be co-transfected into 293T cells. Lentivirus supernatant will be collected from day 2-3 culture

supernatants followed by ultracentrifuge (120,000 g) to concentrate lentivirus for TIL transduction. 1E5 pre-REP cells will be added into Retronectin pre-coated 48 well plates with concentrated lentivirus. Two days after gene transduction, pre-REP cells will be harvested for REP process or further phenotypic characterization and functional assays.

TABLE 22. Membrane anchored IL-12, IL-15 and IL-21 DNA sequences (with NFAT Promoter)

| Construct                   | DNA sequence                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|-----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NFAT-<br>Te-IL12<br>DNA seq | <u>tcgaggtcgacggtatcgataagcttgatcgaattaggaggaaaaactgtttcacacagaaggcgctcaattag</u><br><u>gaggaaaaactgtttcacacagaaggcgctcaattaggaggaaaaactgtttcacacagaaggcgctcaattggtcc</u><br><u>catcgaattaggaggaaaaactgtttcacacagaaggcgctcaattaggaggaaaaactgtttcacacagaaggc</u><br><u>gtcaattaggaggaaaaactgtttcacacagaaggcgctcaattggtccgggacatttgcacccccataaatl</u><br><u>ttccagaattaacagtataaattgcatctctgttcaagagttccctatcactctcttaatcactactcacagtaacct</u><br><u>caactcctggccacc</u> ATGGATTGGACCTGGATTCTGTTCTCTCGTGGCCGCCG<br>CTACCCGCGTGCACCTCCATCTGGGAGCTGAAGAAAGACGTGTACGT<br>GGTGGAATTGGATTGGTACCCCGACGCCCCAGGCGAGATGGTGGTT<br>CTGACTTGCGACACGCCGGAGGAGGACGGCATCACCTGGACGCTG<br>GACCAGAGCAGCGAAGTGCTGGGCTCTGGTAAGACCTTAACCATCC<br>AGGTCAAGGAGTTTCGGTGATGCAGGACAGTACACGTGTCACAAGG<br>GCGGGGAGGTCTTGTCCTATAGCCTGCTGCTCCTGCACAAGAAGGA<br>AGACGGCATCTGGTCTACGGATATCCTTAAAGACCAGAAGGAGCC<br>CAAGAACAAAACCTTCCTGCGATGCGAGGCTAAGAATTACTCCGG<br>GCGCTTTACCTGTTGGTGGCTCACACCATCTCGACCGACCTGACC<br>TTCTCCGTGAAGTCTTCCAGGGGATCTTCTGATCCGCAAGGCGTTA<br>CCTGCGGGGCCGCTACCCTGTGCGCTGAGCGCGTCCGTGGTGACAA<br>CAAGGAGTACGAGTACTCGGTGGAATGCCAGGAGGACAGCGCCTG<br>CCCAGCGGCCGAAGAGTCCTTGCCCATCGAGGTGATGGTGGACGCT<br>GTACACAAGCTCAAGTACGAGAACTACACCTCCTCCTTTTATAC<br>GCGACATCATCAAGCCTGACCCCCCTAAAAACCTGCAGCTGAAGCC<br>CCTTAAAAACTCTCGACAGGTTCGAGGTATCCTGGGAGTACCCCGAC<br>ACCTGGTCTACTCCTCATTCGTATTTACGCCTGACCTTCTGCGTGCA<br>GGTGCAGGGCAAGTCCAAGCGCGAGAAGAAGGACCGCGTGTTTAC<br>CGACAAAACCAGCGCGACTGTTCATTTGTCGGAAGAACGCGAGCAT<br>CTCCGTACGGGCACAGGATCGCTATTACTCCAGCAGTTGGTCAGAG<br>TGGGCTTCGGTTCCGTGTTCTGGAGGAGGTGGCGGCGGCTCACGCA<br>ACCTGCCCCTGGCGACGCCGGACCCGGGTATGTTTCCTTGTCTGCA<br>CCACTCTCAGAACTTGCTCCGTGCCGTCTCGAATATGCTGCAGAAG<br>GCCCCGCCAGACTCTGGAGTTCTATCCATGCACTAGTGAGGAGATCG<br>ATCATGAGGACATCACCAAGGACAAGACATCCACCGTGGAGGCTT<br>GTCTGCCGCTGGAGTTGACAAAGAACGAGAGCTGCCTCAATAGCC<br>GCGAGACTTCCTTCATCACCAACGGCTCCTGCCTCGCGAGCCGTAA<br>GACCTCCTTTATGATGGCGCTGTGCCTGTCAAGTATCTACGAAGAT<br>CTGAAAATGTATCAGGTGGAGTTCAAACTATGAACGCAAACTCC<br>TGATGGATCCCAAGCGCCAAATTTTCTTGACCAGAACATCGCTTGC<br>CGTGATCGACGAGCTGATGCAGGCCCTGAACCTCAACTCCGAGACC<br>GTCCCTCAGAAGTCGTCTCTGGAGGAACCCGACTTTTACAAGACAA<br>AGATCAAGCTGTGCATCCTGCTACACGCGTTCCGCATTCGGGCCGT |

|                             |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                             | GACCATTGACCGCGTGATGTCCTACCTGAATGCCTCGTCCGGCGGG<br>GGGGGCTCCGGTGGGGGCGGCTCTGGCGGAGGCGGCTCAGGTGGC<br>GGCGGCTCCGGCGGAGGTTTCGCTTCAGCTTCTGCCCAGTTGGGCAA<br>TCACTCTGATTTCCGTCAACGGAATCTTCGTATCTGCTGCCTGACC<br>TACTGCTTTGCTCCCAGATGTAGAGAGCGCCGCCGCAACGAGCGCC<br>TGCCTCGTGAGAGCGTGAGGCCTGTGTGA<br>(SEQ ID NO:156)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
| NFAT-<br>Te-IL15<br>DNA seq | <u>tcgaggtcgacggtatcgataagcttgatatcgaattaggaggaaaaactgtttcatacagaaggcgtaattag</u><br><u>gaggaaaaactgtttcatacagaaggcgctcaattaggaggaaaaactgtttcatacagaaggcgtaattggtcc</u><br><u>catcgaattaggaggaaaaactgtttcatacagaaggcgctcaattaggaggaaaaactgtttcatacagaaggc</u><br><u>gtcaattaggaggaaaaactgtttcatacagaaggcgctcaattggtccgggacatttgacacccccataatatt</u><br><u>ttccagaattaacagtataaattgcatctctgttcaagagttccctatcactctcttaatactactcacagtaacct</u><br><u>caactcctggccacc</u> ATGGACTGGACTTGGATACTCTTTCTGGTCGCTGCCG<br>CCACACGGGTGCACTCTAATTGGGTCAACGTGATCAGCGACCTGAA<br>GAAGATCGAGGACCTGATCCAGAGCATGCACATCGACGCCACACT<br>GTACACCGAGTCCGATGTGCACCCTAGCTGCAAAGTGACCGCCATG<br>AAGTGCTTTCTGCTGGAAGTGC AAGTGATCAGCCTGGAAAGCGGCG<br>ACGCCAGCATCCACGATACCGTGGAATCTGATCATCCTGGCCAA<br>CAACAGCCTGTCCAGCAACGGCAATGTGACCGAGAGCGGCTGCAA<br>AGAGTGCGAGGAACTGGAAGAAAAGAACATCAAAGAGTTTCTGCA<br>GAGCTTCGTCCACATCGTGCAGATGTTCATCAACACCTCATCAGGT<br>GGCGGTGGAAGCGGAGGTGGCGGTAGTGGCGGCGGAGGCTCAGGC<br>GGCGGAGGTTCCGGCGGAGGATCTCTTCAGCTCCTGCCATCTTGGG<br>CTATCACCTGATTAGTGTGAATGGGATCTTTGTCATCTGTTGTCTC<br>ACGTA CTGTTTCGCTCCCCGGTGCAGAGAGAGAAGGCGCAACGAA<br>AGACTGCGGAGAGAAAGCGTCAGACCCGTGTGA (SEQ ID NO:157)                                                                                                                                      |
| NFAT-<br>TeIL21<br>DNA seq  | <u>tcgaggtcgacggtatcgataagcttgatatcgaattaggaggaaaaactgtttcatacagaaggcgtaattag</u><br><u>gaggaaaaactgtttcatacagaaggcgctcaattaggaggaaaaactgtttcatacagaaggcgtaattggtcc</u><br><u>catcgaattaggaggaaaaactgtttcatacagaaggcgctcaattaggaggaaaaactgtttcatacagaaggc</u><br><u>gtcaattaggaggaaaaactgtttcatacagaaggcgctcaattggtccgggacatttgacacccccataatatt</u><br><u>ttccagaattaacagtataaattgcatctctgttcaagagttccctatcactctcttaatactactcacagtaacct</u><br><u>caactcctggccacc</u> ATGGATTGGACCTGGATTCTGTTCTCTGGTGGCCGCTG<br>CCACAAGAGTGCATAGCCAGGGCCAAGACCGGCACATGATCCGGA<br>TGAGACAGCTGATCGACATCGTGGACCAGCTGAAGAACTACGTGA<br>ACGACCTGGTGCCTGAGTTCCTGCCTGCTCCTGAGGACGTGGAAAC<br>AAATTGCGAGTGGTCCGCCTTCAGCTGCTTCCAGAAGGCCAGCTG<br>AAAAGCGCCAACACCGGCAAC AACGAGCGGATCATCAACGTGTCC<br>ATCAAGAAGCTGAAGCGGAAGCCTCCTAGCACCAATGCCGGAAGA<br>AGGCAGAAGCACAGACTGACCTGTCTTAGCTGCGACAGCTACGAG<br>AGAAGCCTCCAAAAGAGTTCTTGAACGGTTCAAGAGCCTGCTG<br>CAGAAGATGATCCACCAGCACCTGAGCAGCAGAACCCACGGCTCT<br>GAAGATTCTAGCGGAGGCGGAGGAAGTGTTGGCGGAGGTTCTGCT<br>GGCGGTGGATCAGGCGGTGGCGGATCTGGCGGCGGAGGCTAGTGGC<br>GGAGGTGGAAGCGGTGGTGGTGGCTCTGGCGGAGGCGGTAGCGGC<br>GGAGGCGGATCTCTTCAATTGCTGCCTAGCTGGGCCATCACACTGA<br>TCTCCGTGAACGGCATCTTCGTGATCTGCTGCCTGACCTACTGCTTC<br>GCCCCTAGATGCAGAGAGCGGAGAAGAAACGAGCGGCTGAGAAG<br>AGAATCTGTGCGGCCTGTTTAA (SEQ ID NO:158) |

|                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|--------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| NFAT-<br>TelIL15-<br>IL21<br>DNA seq | <u>tcgaggtcgacggtatcgataaagcttgatatcgaattaggaggaaaaactgtttcatacagaaggcgtcaattag</u><br><u>gaggaaaaactgtttcatacagaaggcgtcaattaggaggaaaaactgtttcatacagaaggcgtcaattgggcc</u><br><u>catcgaattaggaggaaaaactgtttcatacagaaggcgtcaattaggaggaaaaactgtttcatacagaaggc</u><br><u>gtcaattaggaggaaaaactgtttcatacagaaggcgtcaattgtccgggacatttgacacccccataatattt</u><br><u>ttccagaattaacagtataaattgcatctctgttcaagagttccctatcactctctttaatcactactcacagtaacct</u><br><u>caactcctggccacc</u> ATGGATTGGACCTGGATTCTGTTCTGTTGGCCGCTG<br>CCACAAGAGTGCATAGCCAGGGCCAAGACCGGCACATGATCCGGA<br>TGAGACAGCTGATCGACATCGTGGACCAGCTGAAGAAGTACGTGA<br>ACGACCTGGTGCCTGAGTTCCTGCCTGCTCCTGAGGACGTGGAAAC<br>AAATTGCGAGTGGTCCGCCTTCAGCTGCTTCCAGAAGGCCAGCTG<br>AAAAGCGCCAACACCGGCAACAACGAGCGGATCATCAACGTGTCC<br>ATCAAGAAGCTGAAGCGGAAGCCTCCTAGCACCAATGCCGGAAGA<br>AGGCAGAAGCACAGACTGACCTGTCCTAGCTGCGACAGCTACGAG<br>AAGAAGCCTCCAAAAGAGTTCCTGGAACGGTTCAAGAGCCTGCTG<br>CAGAAGATGATCCACCAGCACCTGAGCAGCAGAACCCACGGCTCT<br>GAAGATTCTAGCGGAGGCGGAGGAAGTGGTGGCGGAGGTTCTGGT<br>GGCGGTGGATCAGGCGGTGGCGGATCTGGCGGCGGAGGCAGTGGC<br>GGAGGTGGAAGCGGTGGTGGTGGCTCTGGCGGAGGCGGTAGCGGC<br>GGAGGCGGATCTCTTCAATTGCTGCCTAGCTGGGCCATCACACTGA<br>TCTCCGTGAACGGCATCTTCGTGATCTGCTGCCTGACCTACTGCTTC<br>GCCCCTAGATGCAGAGAGCGGAGAAGAAACGAGCGGCTGAGAAG<br>AGAATCTGTGCGGCCTGTTAGAGCCAAGAGATCTGGAAGCGGCGC<br>CACCAACTTTAGCCTGCTGAAACAGGCTGGCGACGTGGAAGAGAA<br>CCCTGGACCTATGGACTGGACTTGGATACTCTTTCTGGTCGCTGCCG<br>CCACACGGGTGCACTCTAATTGGGTCAACGTGATCAGCGACCTGAA<br>GAAGATCGAGGACCTGATCCAGAGCATGCACATCGACGCCACACT<br>GTACACCGAGTCCGATGTGCACCCTAGCTGCAAAGTGACCGCCATG<br>AAGTGCTTTCTGCTGGAAGTGAAGTATCAGCCTGGAAAGCGGCG<br>ACGCCAGCATCCACGATACCGTGGAATCTGATCATCCTGGCCAA<br>CAACAGCCTGTCCAGCAACGGCAATGTGACCGAGAGCGGCTGCAA<br>AGAGTGCGAGGAACTGGAAGAAAAGAACATCAAAGAGTTTCTGCA<br>GAGCTTCGTCCACATCGTGCAGATGTTTCATCAACACCTCATCAGGT<br>GGCGGTGGAAGCGGAGGTGGCGGTAGTGGCGGCGGAGGCTCAGGC<br>GGCGGAGGTTCCGGCGGAGGATCTCTTCAGCTCCTGCCATCTTGGG<br>CTATCACCTGATTAGTGTGAATGGGATCTTTGTCATCTGTTGTCTC<br>ACGTAAGTGTTCGCTCCCCGGTGCAGAGAGAGAAGGCGCAACGAA<br>AGACTGCGGAGAGAAAGCGTCAGACCCGTGTAA (SEQ ID NO:159) |
|--------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

[00576] Flow cytometry

[00577] To check mIL-15 expression in transduced cells, biotin-conjugate IL-15 (biolgend, San Diego, CA) plus streptavidin-BV421 (Biolegend, San Diego, CA) will be used to stain transduced cells. To check mIL-21 expression, PE-conjugated IL-21 antibodies will be used to stain transduced cells. To check T cell division, CellTrace™ Violet Cell Proliferation dye (ThermoFisher scientific, Waltham, MA) will be used to pre-label T cells. T

cell division was analyzed after 5 days in ex vivo culture in presence or absence of IL-2 (20IU/ml). To check T cell activation and differentiation, antibodies from BD Bioscience, Biolegend, Thermofisher will be used for phenotypic characterization. Data was acquired with a BioRad ZE5 flow cytometer and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR).

**[00578]**      T cell count and viability

**[00579]**      REP cells will be harvested after TIL expansion. Live cell numbers will be assessed by AOPI assay using Cellometer K2 cell counter machine (Nexcelom, Lawrence, MA).

**[00580]**      Pre-REP and REP process

**[00581]**      Human tumor samples will be dissected into ~3 mm pieces and cultured in Grex10 with recommended culture medium containing IL-2 (6000IU/ml). After 11 days of culture, pre-REP cells will be harvested for 2-day activation and 2-day lentiviral transduction. Transduced Pre-REP cells will be then propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and 3,000 IU/ml of IL-2 for another 11 days.

**[00582]**      **Results**

**[00583]**      Expression of mIL-15/mIL-21 after gene transduction

**[00584]**      After gene transduction, transduced pre-REP cells will be ex vivo expanded with 300IU/ml IL-2 for another 5 days. By flow cytometry staining, transduced pre-REP cells will be assessed for expression of mIL-15 and mIL-21. Un-transduced cells will be used as negative control.

**[00585]**      mIL-15 pre-REP cells exhibit sustained activation of pSTAT5 signaling and showed increased proliferation

**[00586]**      After verification of surface cytokine expression, T cell functionality will be examined. In particular, phosphorylation of STAT5 of the modified TILs will be assessed under serum starved conditions to determine if the modified TILs express function mIL-15 and/or IL-21. Further, cell proliferation of mIL-15 lentiviral transduced TILs will be examined by CellTrace proliferation assay.

**[00587]**      Expression of mIL-15/mIL-21 after REP process

[00588] After lentiviral transduction, TILs will be expanded for 11 days in a REP process and surface expression of membrane-bound cytokine receptors will then be assessed by flow cytometry. Harvested REP cells will be assessed for CD8+ T cells. Previous reports show that IL-15 and IL-21 act as a potent stimulator, which preferentially stimulates the proliferation and survival of CD8 T cells.

[00589] Phenotype of mIL-15/mIL-21 expressing REP TIL

[00590] To characterize the immune regulatory effect of mIL-15 and mIL-21 on REP cells, phenotypic analysis will be performed with freshly thawed REP cells in both gated CD8+CD3+T cells and CD4+CD3+ T cell subset. Expression of the following will be assessed: CD27, PD1, TIM3, CD62L, TOX, T-bet, CD38, CXCR3, Eomes, and CD25.

**EXAMPLE 6: PRODUCTION AND CHARACTERIZATION OF MODIFIED TILs THAT EXPRESSED TEHTERED IL-12 UNDER THE CONTROL OF AN NFAT PROMOTER AND REDUCED EXPRESSION OF PD-1**

[00591] For this study, modified TILs with membrane anchored IL-12 immunomodulatory fusion proteins and one or more shRNAs for reducing endogenous PD-1 expression will be made. Figure 5 depicts an exemplary nucleic acid construct that allows for expression of the member anchored IL-12 (TeIL-12) and PD-1 shRNA. As shown in Figure 5, expression of the membrane anchored immunomodulatory IL-12 is controlled using an NFAT promoter that includes NFAT responsive elements linked to a minimal human IL-2 promoter, see, e.g., Table 23. The shRNA is under the control of a PolIII promoter (U6).

Virus preparation and T cell transduction

[00592] To make lentivirus, pLenti-vectors containing the tethered cytokine gene sequence (Table 23) and PD-1 shRNA (Table 9) and packaging helper vectors (BaEV-TR, Gag/Pol) will be co-transfected into 293T cells. Lentivirus supernatant will be collected from day 2-3 culture supernatants followed by ultracentrifuge (120,000 g) to concentrate lentivirus for TIL transduction. 1E5 pre-REP cells will be added into Retronectin pre-coated 48 well plates with concentrated lentivirus. Two days after gene transduction, pre-REP cells will be harvested for REP process according to the methods described herein or further phenotypic characterization and functional assays.

TABLE 23 – Sequences for TeIL-12/shRNA constructs

[illegible]

|                |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| IRES           | CCCCTCTCCCTCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGAAT<br>AAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCCTT<br>TTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATT<br>CCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGT<br>CGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCT<br>GTAGCGACCCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCC<br>TCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACA<br>ACCCACAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGG<br>CTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTAC<br>CCCATTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGT<br>GTTTAGTCGAGGTTAAAAAACGTCTAGGCCCCCGAACCACGGGGACG<br>TGGTTTTCCTTTGAAAAACACGATGATAA (SEQ ID NO:163) |
| U6<br>promoter | GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGC<br>TGTTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTA<br>GTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGT<br>TTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAA<br>AGTATTTGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC<br>(SEQ ID NO:164)                                                                                                                                                                                                                                                                                                                                                               |

Flow cytometry

[00593] TIL TeIL-12 and PD-1 expression will be assessed using immunofluorescence staining and flow cytometric analysis.

T cell count and viability

[00594] REP cells will be harvested after TIL expansion. Live cell numbers will be assessed by AOPI assay using Cellometer K2 cell counter machine (Nexcelom, Lawrence, MA).

Pre-REP and REP process

[00595] Human tumor samples will be dissected into ~3 mm pieces and cultured in Grex10 with recommended culture medium containing IL-2 (6000IU/ml). After 11 days of culture, pre-REP cells will be harvested for 2-day activation and 2-day lentiviral transduction. Transduced Pre-REP cells will be then propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and 3,000 IU/ml of IL-2 for another 11 days.



**EXAMPLE 7: PRODUCTION AND CHARACTERIZATION OF MODIFIED TILs THAT EXPRESSED TETHERED IL-12 UNDER THE CONTROL OF AN NFAT PROMOTER**

[00596] Fig. 6 shows an exemplary TIL expansion process including transduction of a lentiviral vector comprising a nucleotide sequence encoding TeIL-12 controlled by either an EF-1alpha promotor or an NFAT promoter that includes NFAT responsive elements linked to a minimal human IL-2 promoter.

[00597] After 11 days of pre-REP in cell culture media including 6000 IU/mL IL-2, TILs were stimulated for 2 days with TransACT at a ratio of 1:100, in the presence of 10 ng/mL IL-7, and 20 ng/mL IL-15.

[00598] Spin transduction was performed with Lentivirus+ at 1:100 Lentiboost-P in CM2 + IL-7 and IL-15 in a RetroNectin® coated plate. After the transduction, TILs were then rested for 2 days, followed with a REP process with 25K TILs, 5E6 Feeder cells in CM2 + IL-2 at 3000 IU/mL + OKT3 at 30 ng/mL (total vol 4 mL) in GREX-24. After 5 days of incubation, an additional 4 mL of CM2 + 3000 IU/mL IL-2 was added to each well. Incubation continued for a total of 11 days followed by TIL harvest.

[00599] After REP harvest, surface expression of IL-12 on TILs was examined by flow cytometry with IL-12P70 flow Ab (Fig. 7A). TILs were also stimulated with TransACT or PMA. 48 hours after stimulation, surface expression of IL-12 was examined by flow cytometry (Fig. 7B).

[00600] TeIL-12 expressing TILs were also assayed for IL-12 activity (Fig. 8). 5E4 HEK-IL18 reporter cells were seeded in a 96-well plate in DMEM + 10% FBS media. After adherence, transduced TILs were seeded at a 5:1, 1:1 and 0.5:1 ratio at 2.5E5, 5E4, and 2.5E4 cells/well, respectively. Transwell was set. After overnight incubation, 20 µl culture supernatant was collected and transferred to another 96 well plate. 180 µL of Quantibblue Reagent was added to each well and incubated at 37°C until the highest IL-12 standard turned into a dark purple color.

[00601] Five tumor samples (2 lung, 1 head & neck, 1 breast, and 1 ovarian) were used to generate TILs for this study. Fig. 9 shows fold expansion (A) and viability (B) of post-REP TILs. Fig. 10 shows expression frequency of TeIL-12 (A) and viral copy number per cell (B) detected by digital PCR.

**[00602]** Cytotoxicity of TeIL-12 expressing TILs was assessed by KILR® THP-1 assay. Post-REP-TILs (1E5) and KILR-THP-1 cells (1E4) were co-cultured in 96 well plates in 10:1 ratio. 24 hours after incubation, supernatant was harvested for cytotoxicity assay. THP-1 target cell death was detected using the KILR detection reagent (Figs. 11A & B). IFN- $\gamma$  production (Figs. 11C & D) and IL-12 shedding in culture supernatant were examined by ELISA (Fig. 11E). Both TeIL-12 and NFAT-driven inducible TeIL-12 TILs demonstrated improved killing activity in a THP-1 based allogeneic cytotoxicity assay. > 30 folds increase of IFN- $\gamma$  was detected in the co-culture assay. TeIL-12 shedding happened during the co-culture of TeIL-12 TILs and KILR® THP-1 cells.

**[00603]** Cytotoxicity of TeIL-12 expressing TILs was also assessed by xCelligence RTCA assay. 10,000 target cells #1 were seeded in an RTCA E-plate. After overnight incubation, post-REP-TILs were added at a 10:1 or 3:1 ratio. Cell growth was dynamically monitored by an xCELLigence RTCA instrument (Fig. 12A). The same assay was performed with target cell #2 (Fig. 12B). TeIL-12 and NFAT TeIL-12 TILs demonstrated superior allogeneic cytotoxicity.

**[00604]** To test serial killing capability, post-REP-TILs were stimulated repeatedly with TransACT (1:100) and harvested 2 days after the last TransACT stimulation (Fig. 13A). KILR® THP-1 cytotoxicity assay, IFN- $\gamma$  quantification (Fig. 13B), and xCELLigence RTCA killing assay (Fig. 13C) were performed to evaluate TIL killing efficacy.

**[00605]** CD8+, CD4+, and CD4+FoxP3+ T cell subsets of post-REP TILs were analyzed by flow cytometry (Fig. 14). No difference was observed on the distribution of CD8+, CD4+, and CD4+FoxP3+ T cell subsets.

**[00606]** Tcm (CCR7+CCR45RA-), Tem (CCR7-CD45RA-), Tema (CCR7-CD45RA+) subsets and expression of CD62L of post-REP TILs were detected by flow cytometry (Fig. 15). No statistic significant difference was observed in Tcm, Tem and Tema distribution. TeIL-12/NFAT-TeIL-12 post-REP TILs showed less differentiation, with increased expression of CD62L.

**[00607]** Expression of T cell exhaustion markers PD-1, LAG-3, Tim-3 and TIGIT on post-REP TILs was detected by flow cytometry (Fig. 16). No significant change of PD-1 and LAG-3 expression was observed. Reduced expression of Tim-3 and TIGIT, more profound in TIGIT, was observed.

[00608] Expression of T cell activation markers CD25, CD38, CD39 and CD69 on post-REP TILs was detected by flow cytometry (Fig. 17). Increased expression of activation marker CD25 was observed in TeIL-12 post-REP TILs (CD8+T cells), while decreased expression of CD25 was observed in NFAT-TeIL-12 post-REP TILs. No change in expression of CD38 was observed, while CD69 expression decreased.

[00609] Expression of T cell function-related markers IFN- $\gamma$ , TNF- $\alpha$ , CD107a and Granzyme B on post-REP TILs was detected by flow cytometry (Fig. 18). Increased IFN- $\gamma$  and Granzyme B production was observed in TeIL-12 post-REP TILs, and more significant in CD8+ T cell population.

#### **EXAMPLE 8: OPTIMIZATION OF ANTI-CD3 ANTIBODY TIMING AND DOSAGE DURING REP AND IMPACT ON EXPANSION AND SURFACE EXPRESSION OF TETHERED IL-15/IL-21**

[00610] This study tested the impact on TIL expansion and surface expression of TeIL-15/TeIL21 by adding an anti-CD3 antibody at different days after the initiation of REP. The above examples are modified as described herein with regard to the timing of the anti-CD3 antibody addition; anti-CD3 antibody is added at the time as indicated in the paragraphs below.

[00611] Pre-REP TILs were prepared using the Gen 2 process described in Example 1, followed by gene transduction with a lentiviral vector comprising a nucleotide sequence encoding TeIL-15 or TeIL-15 and TeIL-21 under the control of an EF-1 $\alpha$  promoter using the protocol below.

##### **Viral Transduction of TILs**

[00612] TILs were thawed by adding 1mL of thawed cell suspension to 9mL pre-warmed CM2 without gentamicin and centrifuge at 500xg, 4min, room temperature (RT).

[00613] Supernatant was discarded and TILs were suspended in 1mL CM2 without gentamicin + 300IU/mL IL-2 and count.

[00614] TILs were suspended at 1e6/mL CM2 without gentamicin + 300IU/mL IL-2 in a 24 well plate at 2mL per well. Incubate overnight.

[00615] A 20x cocktail of IL-15, IL-7 was prepared for the addition of 100uL per well.

- o Final concentrations: IL-15 (10ng/mL), IL-7 (20ng/mL) in CM2 without gentamicin

- Example: 10 wells needed
  - 10 wells x 100uL cocktail = 1mL
    - Add IL-15 to a concentration of 200ng/mL
    - Add IL-7 to a concentration of 400ng/mL
- Note: Remove 120uL from each well before adding TransACT, IL-15, and IL-7 so that the final volume is 2mL

**[00616]** 100uL of 20x cytokine cocktail was added to each TIL culture within the 24 well plate. Then, 20uL TransACT was added to each well. Each well was pipette mixed gently to evenly disperse TILs and to mix with stimulation cocktail. Incubate for 2 days.

**[00617]** A Non-tissue culture 48 well plate with Retronectin (diluted 1:100 in PBS) was coated by adding 250uL per well. Wrap plate in parafilm and then incubate at 4°C overnight.

**[00618]** The coating solution was removed from retronectin plate and replace with 250uL 2% BSA Fraction V. Note: The 2% BSA Fraction V was added immediately so that the well did not dry out.

**[00619]** Incubated at RT for 30min for blocking.

**[00620]** Blocking solution was removed. The calculated volumes of viral supernatant were added and wrapped in parafilm. Centrifuged for 2000xg, 90min, 32 °C. Note: Centrifuge was warmed prior to adding plate.

**[00621]** While the plate was spinning with virus, TILs were collected and counted. TILs were suspended at 1e6/mL in CM2 without gentamicin.

**[00622]** Once the plate finished spinning, the following was added:

5. 100uL of TIL suspension (1e5 cells)
6. 200uL CM2 without gentamicin
7. 300uL CM2 + IL-15 (20ng/mL) + IL-7 (40ng/mL) + Lentibooster 1:50
8. = Total 600uL per well

**[00623]** Centrifuged 2000xg, 20min, 32 °C. Placed in incubator and incubate for 3 days.

**[00624]** After transduction of Pre-REP TILs with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule capable of expressing TeIL-15, Pre-REP TILs were processed for REP expansion with feeder cell, 3000 IU/ml IL-2 and an anti-CD3 antibody, OKT3 or HIT3a. OKT3 (30ng/ml) or HIT3a (30ng/ml) was added into the REP culture medium on different days (Day 0, Day 2, and Day 4) after starting the REP process. After 11

days of REP expansion, post-REP TILs were harvested. TIL expansion, and surface expression of TeIL-15/TeIL-21 were analyzed using the protocol below. Adding OKT3 or HIT3a on Day 2 or Day 4 led to increased surface expression of TeIL-15 (Fig. 19A and B).

**[00625] Surface expression check for tethered cytokine (2X dilution with 1E5)**

**[00626] Maker 20 sample**

**[00627]** 25ul/sample X10 487.5ul DPBS

**[00628]** Live/dead 1:200 2.5ul

**[00629]** FC block 1:50 10ul

**[00630]** 8mins @RT

**[00631] 50 staining buffer**

**[00632]** 50ul staining buffer 20 sample 860ul

**[00633]** CD3 BUV737 UCHT1 1.5ul 30ul

**[00634]** CD45 PerCP HI30 1ul 20ul

**[00635]** IL-21 PE 4BG1 2.5ul 50ul

**[00636]** IL15 Biotin BH1543 2ul 40ul

**[00637]** Ab incubate 45mins @4C, wash twice

**[00638]** 50ul staining buffer 20 samples 992ul

**[00639]** Avidin-BV421 1:200 0.25ul 5ul

**[00640]** Ab incubate 20mins @4C, wash twice

**[00641]** Add 200ul Staining buffer, for flow cytometry run

**[00642]** After transduction of Pre-REP TILs with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule capable of expressing TeIL-15 and TeIL-21, Pre-REP TILs were processed for REP expansion with feeder cell, 3000 IU/ml IL-2 and an anti-CD3 antibody, OKT3 or HIT3a. OKT3 (30ng/ml) or HIT3a (30ng/ml) was added into the REP culture medium on different days (Day 0, Day 2, and Day 4) after starting the REP process. After 11 days of REP expansion, post-REP TILs were harvested. TIL expansion, and surface expression of TeIL-15/TeIL-21 were analyzed using the protocol above. Adding OKT3 or

HIT3a on Day 2 or Day 4 led to increased surface expression of TeIL-15 and TeIL-21 (Fig. 20A and 20B).

**[00643]** After transduction of Pre-REP TILs with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule capable of expressing TeIL-15, Pre-REP TILs were processed for REP expansion with feeder cell, 3000 IU/ml IL-2 and an anti-CD3 antibody, OKT3. OKT3 was added into the REP culture medium on Day 0 at different concentrations (30 ng/mL, 10 ng/mL, 5 ng/mL, 3 ng/mL, and 1 ng/mL). After 11 days of REP expansion, post-REP TILs were harvested. TIL expansion, and surface expression of TeIL-15/TeIL-21 were analyzed using the protocol above. Adding OKT3 at 30 ng/mL led to the highest surface expression of TeIL-15 (Fig. 21A and 21B).

**[00644]** After transduction of Pre-REP TILs with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule capable of expressing TeIL-15 and TeIL-21, Pre-REP TILs were processed for REP expansion with feeder cell, 3000 IU/ml IL-2 and an anti-CD3 antibody, OKT3. OKT3 was added into the REP culture medium on Day 0 at different concentrations (30 ng/mL, 10 ng/mL, 5 ng/mL, 3 ng/mL, and 1 ng/mL). After 11 days of REP expansion, post-REP TILs were harvested. TIL expansion, and surface expression of TeIL-15/TeIL-21 were analyzed using the protocol above. Adding OKT3 at all concentrations led to low surface expression of TeIL-15 and TeIL-21 (Fig. 22A and 22B).

#### **EXAMPLE 9: OPTIMIZATION OF TRANSDUCTION CONDITIONS FOR TeIL-12 EXPRESSION**

**[00645]** Pre-REP TIL was activated with or without TransACT, then transduced with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule capable of constitutive expression of TeIL-12, and then subjected to REP process. Surface expression of TeIL-12 was examined by flow assay (Fig. 23).

**[00646]** Activated Pre-REP TILs were transduced in retronection or vectofusin coated plates with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule capable of constitutive expression of TeIL-12, with or without centrifugation. Surface expression of TeIL-12 was examined by flow assay after REP expansion. Transduction with Retronection coated plate resulted in better transduction efficiency than transduction with Vectofusin coated plate (Fig. 24). Transduction without centrifugation reduced transduction efficiency (Fig. 24).

[00647] Lentiboost was added during spin transduction of activated Pre-REP TILs with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule encoding NFAT-TeIL-12. Transduction efficiency was evaluated by surface TeIL-12 expression check upon TransACT or PMA-Inomycin stimulation. Transduction with or without Lentiboost showed similar TeIL-12 surface expression (Fig. 25).

[00648] Lentiboost was added during spin transduction of activated Pre-REP TILs with a lentiviral vector encapsulating a recombinant lentiviral RNA molecule encoding NFAT-TeIL-12. Transduction efficiency was evaluated by surface TeIL-12 expression check upon TransACT or PMA-Inomycin stimulation. Transduction using the BaEVTR Env plasmid resulted in more TeIL-12 surface expression compared to transduction using RD114 or VSV-G Env plasmid (Fig. 26).

#### **EXAMPLE 10: NFAT-TEIL-12 ENGINEERED TIL DEMONSTRATES SUPERIOR CYTOTOXICITY**

[00649] Tumor tissue from several solid tumor types was fragmented and cultured, followed by transduction with lentivirus containing a gene encoding membrane-bound IL-12 (TeIL-12) via an NFAT-promoter, then expanded with a rapid expansion protocol (REP). Expression, biological function, and shedding of IL-12 molecule were assessed in vitro. Immune phenotype and in vitro cytotoxic activity of TeIL-12 gene-engineered TIL were examined in various assays.

[00650] Lentiviral gene transfer resulted in TIL expressing IL-12 on their surface via a membrane anchor. This modification potentiated IL-12 receptor downstream signaling activation in a contact-dependent manner in assays using HEK-IL-12-Blue reporter cells. TeIL-12-TIL exhibited increased IFN- $\gamma$  production and superior cytotoxicity in a KILR<sup>®</sup> assay. An xCELLigence-based cytotoxicity assay further confirmed increased killing with TIL that were unstimulated or stimulated. Moreover, phenotype profiling revealed TeIL-12-TIL were less differentiated, with reduced expression of immune-inhibitory receptors and increased production of cytotoxic molecules associated with anti-tumor activity. IL-12 shedding in the co-culture supernatant was minimal.

[00651] KILR<sup>®</sup> THP-1 cells (1E4) were co-cultured with 5 individual REP-TILs (1E5) engineered with NFAT-TeIL-12, mock, pLV-ctrl and mock control cells. 24hrs later, the supernatant was harvested. Cytotoxicity was then assessed using KILR<sup>®</sup> THP-1 cytotoxicity assay (Fig. 27A), and IFN-g was quantified by ELISA (Fig. 27B).

[00652] Freshly thawed REP-TIL, or 3X times TransACT repeatedly stimulated REP-TIL, were added into target cell (CaOV3) pre-seeded E-Plates. Target cell growth was dynamically monitored by xCELLigence RTCA assay. NFAT-TeIL-12 expressing TILs demonstrated superior cytotoxicity (Fig. 27C) and serial killing efficacy (Fig. 27D).

[00653] The enhanced in vitro killing activity, combined with a less-differentiated phenotype of TIL with inducible and membrane-bound IL-12 suggests a potential for increased clinical efficacy. Further, minimal IL-12 shedding supports reduced potential for IL-12-associated toxicity (Zhang et al., *Clin Cancer Res.* **2015**;21:2278–88).

#### **EXAMPLE 11: TILS ENGINEERED WITH NFAT-TEIL-12 AND IL-15/IL-2, PD-1 SHRNA**

[00654] To improve in vivo expansion capability of NFAT-TeIL-12 TILs and possibly avoid use of Aldesleukin in patients after TIL therapy, NFAT-TeIL-12 TILs were further engineered to co-express IL-15 or IL-2 (with or without membrane anchor) under the control of an EF-1 $\alpha$  promoter (Fig. 28). Co-expression of a PD-1 shRNA was also tested to improve TIL function in the tumor microenvironment (TME). tCD19 was coexpressed under the same EF-1 $\alpha$  promoter, can be used as an expression indicator of IL-15, IL-2 or PD-1 shRNA.

[00655] After lentiviral vector (LV) transduction and 11-days REP expansion, genetically edited TIL was harvested. Live cell count was conducted, and fold expansion was calculated based on initial transduced pre-REP TIL (Fig. 29A); viability was examined in Celica (Fig. 29B).

[00656] Transduction efficiency was accessed by the percentage of TeIL-12+/tCD19+ and TeIL-12-/tCD19+ TILs in unstimulated condition examined by flow assay (Fig. 30A). Viral copy number (VCN) per cell was detected by ddPCR (Fig. 30B).

[00657] TCR signal activator, TransACT (1:1000, 1:100, 1:10) and PMA/Inomycin were used to activate downstream NFAT signaling pathway, which sequentially up-regulate NFAT driven TeIL-12 expression in genetically edited TIL. Meanwhile, tCD19 was used as indicator for the expression of TeIL-2, TeIL-15, IL-2, IL-15 and PD-1 shRNA which was under EF-1 $\alpha$  promoter. Co-staining of surface expression of TeIL-12, and tCD19 (noted as inducible TeIL-12 positive cells in genetically edited TIL) was analyzed by flow cytometry. Percentage of TeIL-12 and tCD19 double positive T (TeIL12+CD19+) was dependent on the level of TCR stimulation (Fig. 31).



[00658] xCELLgene RTCA killing assays were performed to assess the killing efficacy of genetically edited TILs targeting CaOV3 cells at an effector-to-target (E/T) ratio of 0.3:1. TILs engineered with NFAT-TeIL-12 and EF-1 $\alpha$ -TeIL-15 exhibited superior cytotoxicity (Fig. 32).

[00659] Genetically edited TILs were co-cultured with CellTrace-labeled Mock TILs (1:1) overnight in serum-free AIM-V in 96 well U-bottom. After wash, fixation and permeabilization, intracellular staining of phosphorylated STAT5 in gated cells were analyzed by flow cytometry. Meanwhile, either IL-2 or IL-15 treated mock cells was used as positive control of phosphorylated STAT5 staining. TeIL-2 and TeIL-15 acted primarily in cis (Fig. 33).

[00660] Genetically edited TIL cells were labeled with CellTrace Blue and cultured in IL-2-free culture medium. 96 hours later, cell division on tCD19<sup>+</sup> (cytokine transduced) and tCD19<sup>-</sup> (cytokine un-transduced) subpopulations was analyzed based on cell trace. TeIL-2 and TeIL-15 show skewed in cis proliferation consistent with p-STAT5 activation (Fig. 34).

[00661] Gen2 TIL was labeled and followed by stimulation of TILs expressing either soluble IL-15 or TeIL-15. p-stat5 activation was examined by flow on gated Gen2 TIL and TeIL-15 TIL. Data shows TeIL-15 activated T cells in cis manner only (Fig. 54).

[00662] Genetically edited TIL was washed and resuspended in IL-2-free CM2. 5E5 cells/well were added to a GREX 24-well plates. Treatments were separated into the following three groups: 1) TransACT plus IL-2; 2) TransACT alone; 3) No stimulation. Cell count and cell viability was examined in Celica. EF-1 $\alpha$  driven IL-2/IL-15 improved T cell proliferation in the absence of IL-2 (Figs. 35A & 35B).

[00663] PD-1 expression frequency on NFAT-TeIL-12 TILs expressing PD-1 shRNA under titrated TransACT stimulation condition in contrast to NFAT-TeIL-12 TILs expressing GFP shRNA as control group. PD-1 expression gMFI on NFAT-TeIL-12 TILs expressing PD-1 shRNA under titrated TransACT stimulation condition in contrast to NFAT-TeIL-12 TILs expressing GFP shRNA as control group. PD-1 shRNA effectively reduced PD-1 positivity in NFAT-TeIL-12 TILs expressing PD-1 shRNA (Figs. 36A & 36B).

[00664] T cell viability and TVC were dynamically examined in different setting: TransACT stimulation (1:100) plus 3000IU/ml IL-2 (Fig. 37A); 3000IU/ml IL-2 only (Fig. 37B); TransACT stimulation (1:100) only (Fig. 37C); and no TransACT stimulation, no IL-2 (Fig. 37D). Data shows that TeIL-15 facilitates TIL survival *in vitro* in the absence of IL-2.

**EXAMPLE 12: NFAT-TEIL-12 GENE ENGINEERED TIL SHOW IMPROVED IN VIVO EFFICACY IN COMPARISON TO GEN2 PROCESSED TIL WITH M1152 PDX MODEL**

[00665] The M1152 PDX ACT model was used to test the *in vivo* efficacy of NFAT-teIL-12 TILs (see Fig. 38A). 1E6 M1152 tumor cells were inoculated into NOG-hIL2 mice subcutaneously. 26 days later, 7.5E6 R-REP'd TILs using different processes were infused into M1152 mice by i.v route. The various processes are as follows:

- i. Gen2
- ii. pLV-ctrl transduction followed with Gen2
- iii. NFAT-TeIL-12-tCD19 transduction followed with Gen2
- iv. NFAT-TeIL-12 transduction followed with Invigo-T+L-Arg process

[00666] Tumor size and mouse body weight were examined twice every week, until mouse reach the endpoint. On day 7 after TIL infusion, PB was collected for plasma cytokine check (see Fig. 38A).

[00667] In comparison to untreated group, TILs made by both the Gen2 process and the Gen2-pLV-ctrl process showed efficacy with tumor suppression; but tumor still grew progressively (see Fig. 38B). However, NFAT-TeIL-12 gene engineered TILs made by either the Gen2 process or the Invigo-T+L-Argine process resulted in tumor growth control (see Fig. 38B). 3/7 mice showed complete regression in the NFAT-TeIL-12-tCD19 group, and 1/7 mice showed complete regression in the Invigo-T modified-NFAT-TeIL-12 group (see Fig. 38B).

[00668] Dynamic change of body weight (Fig. 39A) and body weight % related to baseline (Fig. 39B) were examined. No significant body weight change was observed across untreated, Gen2 TIL and TeIL-12 TIL treated group after TIL infusion.

[00669] Peripheral blood was collected at Day 7 after TIL infusion. Plasma IFN $\gamma$ , TNF $\alpha$ , CCL4 and IL-12 p70 were examined by Bioplex assay. No circulating IL-12 p70 and TNF $\alpha$  was detected in NFAT-TeIL-12 TIL infused mice. Increased plasma IFN $\gamma$  was observed in NFAT-TeIL-12 TIL treatment group (47.9pg/ml) in comparison to Gen2 control TIL (20.3pg/ml). No significant difference of CCL4 was observed between NFAT-TeIL-12 TIL treatment group (13.92 pg/ml) and Gen2 TIL treatment group (13.27 pg/ml). See Fig. 40.

**EXAMPLE 13: TUMOR DIGEST PRIMING WITH IFN $\gamma$  DRAMATICALLY INCREASED REACTIVITY AFTER THE PRE-REP STAGE**

[00670] Tumors were digested into single cell suspensions and plated at 600k cells in a 96 well flat bottom plate. Cells were then cultured for 11 days under the following conditions: IL2 (6000 IU/mL) control, ITIL cytokines (IL-2 (6000 IU/mL), IL-15 (10 ng/mL) and IL-21 (10 ng/mL), or ITIL cytokines + IFN $\gamma$  (200 ng/ml) or ITIL + anti-PD1 (pembrolizumab) at 10 ug/mL or ITIL cytokines + anti-PD1 + IFN $\gamma$  in complete media. The media was changed every 2-3 days. After 11 days, cells were counted and co-cultured with autologous tumor digest (100k TIL: 100k tumor digest cells) for 24 hours. In order to determine the percentage of tumor digest reactive T cells, cells were stained for the activation marker 4-1BB (Biolegend, Clone: 4B4-1, BV421) and flow cytometry was run on a Bio-Rad ZE5 flow cytometer to quantify the percentage of 4-1BB+ reactive T cells. As shown in Fig. 41, tumor reactivity was enhanced when TILs were cultured with ITIL cytokines in the presence of IFN $\gamma$ .

**EXAMPLE 14: SORT OUT THE TUMOR REACTIVE CD8 TIL USING THE T CELL ACTIVATION MARKER 4-1BB**

[00671] 4-1BB is known to be upregulated in response to TCR triggering, particularly on CD8 T cells, but also on a subset of CD4 T cells. However, 4-1BB is only expressed transiently, so an experiment was conducted to determine the optimal timepoint at which to sort TIL based on 4-1BB expression. In this experiment, tumor digests from five NSCLC tumors and five renal cell carcinoma tumors were plated at 6e5 cells in 96-well flat bottom plates in cell culture media (AIM V) supplemented with IL2 (3000 IU/mL) and IFN $\gamma$  (200 ng/mL). As shown in Fig. 42A, 4-1BB expression was found to be maximal 24 hours after plating the tumor digest, returning to baseline (t=0) levels 48 hours after plating. Thus, 24 hours was identified as a preferable time point for sorting out the tumor-reactive TILs.

[00672] To demonstrate the importance of adding in IFN $\gamma$  to maximize 4-1BB expression on the tumor digest-reactive TIL, tumor digest cells were plated (600k in a 96-well plate) from eight NSCLC tumors in either control media (IL-2 at 3000 IU/mL) or TS-TIL media (IL-2 at 3000 ng/mL, IL-21 at 10 ng/mL + IFN $\gamma$  at 200 ng/mL). After 24 hours, 4-1BB expression on CD8+ TIL was again measured using flow cytometry. As shown in Fig. 42B, 4-1BB expression was found to increase with the TS-TIL condition relative to the control condition (IL-2 only). This experiment highlights the importance of adding IFN $\gamma$  to

media, in order to enhance recovery of tumor digest-reactive TILs via increasing 4-1BB expression. In a separate experiment, post-REP TILs from five patients were plated at 2e6 cells in a 24-well plate for 24 hours in media supplemented with IL-2 with or without IFN $\gamma$ . Notably, IFN $\gamma$  did not cause a non-specific increase in the percentage of 4-1BB<sup>+</sup> CD8 T cells, implying that upregulation is likely in response to increase TCR triggering, as a result of enhanced antigen-presentation mediated by IFN $\gamma$  in the tumor digest (data now shown).

#### **EXAMPLE 15: COMPARISON OF SEVERAL PROCESSES FOR THEIR CAPACITY TO ENRICH FOR TUMOR-REACTIVE CD8 TIL**

**[00673]** Several processes were analyzed for their capacity to enrich tumor-reactive CD8 TILs as outlined in Fig. 43. In all cases, tumor digests were generated as described previously and tumor digests were initially plated at 550k cells in a 96-well plate and expanded in CM2 media with the indicated cytokines.

**IL2 CTRL:** pre-REP=IL-2 at 6000 IU/mL and REP = IL-2 at 3000 IU/mL

**CD103+ Enrichment:** pre-REP=IL-2 at 6000 IU/mL and REP = IL2 at 3000 IU/mL  
OR IL-2 (1000 IU/mL)+ IL-15 (10 ng/mL)+IL-21 (10 ng/mL)

**4-1BB+ Enrichment:** pre-REP= IL-2 (1000 IU/mL)+ IL-15 (10 ng/mL)+IL-21 (10 ng/mL) and REP= IL-2 (1000 IU/mL)+ IL-15 (10 ng/mL)+IL-21 (10 ng/mL)  
(referred to these as “TS-TIL” cytokines).

**[00674]** The media was changed and cells were split every 2-3 days as needed, in order to maintain a population of less than 400k and more than 50k viable cells 3 days after the initial tumor digest plating during the pre-REP stage of expansion. All pre-REP populations were rapidly expanded (REP'd) in a 24-well GREX plate in the presence of 5e5 irradiated donor PBMCs and 30 ng/mL of OKT3 from Biolegend by transferring 25k cells from the preREP population into the REP.

**[00675]** As shown on the left hand side of the process flow diagram of Fig. 43, a bulk TIL control arm was employed. For the control arm, TILs from digests were expanded for 10 days during the pre-REP and then rapidly expanded for 10 days during the REP.

**[00676]** For the CD103 enrichment the same population of Day 10 pre-REP cells was used as the control population. However, on day 10, these cells were flow-sorted into four distinct populations: CD103<sup>+</sup>CD31<sup>+</sup>, CD103<sup>+</sup>CD31<sup>-</sup>, CD103<sup>-</sup>CD31<sup>+</sup> and CD103<sup>-</sup>CD31<sup>-</sup>. These populations were then REP'd individually.

[00677] For the TS-TIL process, cells were plated in TS-TIL cytokines plus IFN $\gamma$  (200 ng/mL) during the first 24 hours. They were then sorted using 4-1BB+ magnetic bead (Miltenyia kit) at 24 hours. The sorted cells were then expanded for 9 additional days in 6e6 T-cell depleted feeders (iPBMCs) during the pre-REP in a 24-well GREX in the presence of TS-TIL cytokines without additional IFN $\gamma$  (1X REP) or alternatively the sorted cells were REP'd immediately. On day 10, both populations were then REP'd in TS-TIL cytokines, so that two distinct populations were generated: 1X REP and 2X REP 4-1BB sorted cells. See right side of Fig. 43.

[00678] On day 20 of the process, upon completion of the REP's all populations were counted, phenotyped for surface marker expression and were analyzed for autologous tumor reactivity using intracellular cytokine staining as follows:

[00679] Autologous tumor reactivity:

[00680] 200k TILs were plated in the presence of 200k digest cells for seven hours in the presence of Monensin (Biolegend), Brefeldin A (Biolegend) and anti-CD107a BUV395 antibody (BD Biosciences). After 7 hours, cells were surface stained for CD8, CD4, CD3 and CD45, then fixed, permeabilize and stained for IFN $\gamma$  (PE) and TNF $\alpha$  (BV650), then cells were analyzed on a Bio-Rad ZE5 cytometer. In order to have proper controls, each population of TIL was assessed under the following conditions:

- 1) TIL+Tumor Digest
- 2) TIL+Tumor Digest+anti-MHC Class I antibody (W6/32) at 100 ug/mL, to account for non-TCR mediated (background) expression of CD107a, IFN $\gamma$  and/or TNF $\alpha$
- 3) TIL alone (negative control)
- 4) TIL+TransAct CD3/CD28 stimulator (positive control)

[00681] Flow data was then gated, processed and analyzed in FlowJo and then plotted in GraphPad Prism. ~10x improvement in product potency was observed even with less cells driven by high percentage of tumor reactive TILs made by the CD137 (4-1BB) sorted process. Note minimal tumor reactivity in CD103-CD31- group, which represents bystanders. See Fig. 44.

**EXAMPLE 16: BIVARIATE FLOW PLOT DATA (IFN $\gamma$  AND CD107A) FROM THE INTRACELLULAR CYTOKINE TUMOR DIGEST**

[00682] The bivariate flow plot data (IFN $\gamma$  and CD107a) was analyzed from the intracellular cytokine tumor digest as previously described. The plots are gated on live CD8+CD3+ T cells “CD8 TIL”. The results are for GEN2 (left) and 4-1BB Sort/1x REP “TS-TIL” (right). See Fig. 45. The upper plots are for TIL-cocultured with autologous tumor digest. The lower plots are for the same experimental setup, except MHC Class I blocking antibody (W6/32) has been added, in order to account for any non-TCR mediated (background) expression of the effector cytokine IFN $\gamma$  or the degranulation marker CD107a. Minimal reactivity is observed in the lower plots due to the presence of MHC Class I Blocking antibody, as expected as this blocks CD8 TCR stimulation by tumor antigens. Very little tumor-specific reactivity is found in CD8 TIL generated under the control (GEN2/bulk TIL) process with only 0.58% of CD8 TIL co-expressing CD107a+ IFN $\gamma$ +. Notably, reactivity is much stronger in the TS-TIL (4-1BB sort/1x REP) process, whereby 11.7% of cells were found to co-express CD107a and IFN $\gamma$ , a nearly 20-fold improvement. The single positive populations (IFN $\gamma$ + or CD107a+) also increased under the TS-TIL process. See Fig. 45. This shows enrichment for tumor reactive TILs made by the TS-TIL (4-1BB sort/1x REP) process.

**EXAMPLE 17: ANALYZE TUMORS FROM 3 ADDITIONAL NSCLC PATIENTS**

[00683] Tumors from 3 additional NSCLC patients L4373, L4375 and L4397 were analyzed. Multiple REP conditions (IL2 at 3000 IU/mL, CTRL) and a test condition referred to as “Invigo-T” (IL-15 (10 ng/mL) + IL-21 (10 ng/mL) + AKTi (AZD4673) at 1  $\mu$ M) were tested to see how this would impact expansion and tumor-reactivity of the final (post-REP) TIL product. The 4-1BB sort/1x REP condition previously described was used.

[00684] The GEN2 TIL control condition vs the TS-TIL 4-1BB sort/1x REP “TS-TIL” condition vs CD103 post pre-REP sort were tested in the three NSCLC patients. For 4-1BB sorting, the impact of the aforementioned REP conditions on CD8 tumor specific reactivity was tested. As before, CD8 tumor digest-reactivity was assessed by autologous digest coculture ICS. The results are shown in Fig. 46 as a function of both sort conditions and REP condition. Both CD137 (4-1BB) sorting conditions provided the best enrichment for tumor-reactive CD8 (IFN $\gamma$  +CD107a+) TILs in the TIL product. CD103 sorting showed an improvement over GEN2 bulk TIL control as well.

**[00685]** To test the impact of these process changes on enrichment for tumor-reactive TILs, the bivariate flow plots for L4397 was analyzed. See Fig. 47. Under GEN2 bulk TIL conditions only 0.24% of CD8 TILs were IFN $\gamma$ +CD107+, whereas under 4-1BB sort/IL2 3000 IU/mL REP conditions, 15.3% of CD8 TILs were IFN $\gamma$ +CD107a+.

**[00686]** As an additional example, a primary NSCLC tumor from Patient L4463 was digested and TIL products were generated using either the TS-TIL process, “TS-TIL” (4-1BB selection) or the Control Process “CTRL” (bulk, unselected TIL expanded with IL-2 only) from only 550k viable digest cells. As shown in Fig. 48, the total process time to generate the final TIL product is 20 days. To compare tumor-specific reactivity from each day 20 product, 200k TIL were co-cultured with 200k autologous tumor digest cells for 7 hours in a 96-well flat plate in 250  $\mu$ L of AIM V media supplemented with 300 IU/mL of IL-2, brefeldin A and monensin. Then cells were stained for CD3, CD45, CD8, CD4, CD107a (degranulation marker) and IFN $\gamma$  (effector cytokine) using intracellular cytokine staining methods, and expression was measured using a ZE5 flow cytometer from Bio-Rad. Tumor-specific reactivity was measured in the CD8 population by defining tumor-reactive TIL as those expressing MHC Class-I dependent co-expression of CD107a and IFN $\gamma$ . Specifically, for complete analysis with appropriate controls, live IFN $\gamma$ +CD107a+CD8+ TILs were measured under the following five conditions:

- A) 200k TIL+ 200k Autologous Digest
- B) 200k TIL+ 200k Autologous Digest+anti-MHC Class I blocking antibody (Clone W6/32, Biologend) at 100  $\mu$ g/mL (background subtraction)
- C) 200k TIL alone (negative control)
- D) 200k TIL+ the CD3/CD8 agonist TransAct (Miltenyi Biosciences, 130-111-160) at 1:20 dilution (positive control)
- E) 200k TIL+CEF peptide mix (1:100), (Miltenyi Biosciences, 130-098-426), (used to assess viral reactivity in TIL).

**[00687]** Condition B was used to measure non-specific (i.e, non peptide:MHC Class I mediated) expression of CD107a and IFN $\gamma$ . These results are shown in Fig. 49.

**[00688]** The specific tumor reactivity was computed as the percentage of digest-reactive TIL in Condition A minus the percentage of digest-reactive TIL in Condition B. TIL alone was used as a negative control (no TCR stimulation present) and TIL + TransAct was used as a positive control, as TransAct is a potent T cell stimulation compound, known to trigger the T Cell Receptor (TCR) and the costimulatory receptor (CD28) and induce secretion of IFN $\gamma$  and CD107a. Further, TILs were cultured with the CEF peptide mix which

contains multiple known MHC Class I epitopes from viral antigens (CMV, EBV, flu) known to be recognized by T cells across multiple HLA types.

**[00689]** The data shows a significant increase in percentage of specific tumor reactive TILs in the TS-TIL product (~7%) vs the CTRL product (~1%). See Fig. 49. Both products showed minimum reactivity when rested alone, as expected due to the absence of TCR stimulation and high reactivity when exposed to the potent TCR stimulus TransAct. Notably, the CTRL TIL product showed a higher percentage of TILs reactive to the MHC Class I peptide mix, which is consistent with the hypothesis that unselected CTRL TILs contain a higher percentage of “bystander” viral-specific TILs, which would not be expected to mediate tumor regression due to the inability to specifically recognize tumor cells.

**[00690]** This same process was repeated for an additional five NSCLC patients and the results are shown in Fig. 50. To calculate the total number of CD8 tumor-reactive TILs generated in each process from 550k digest cells, following formula was used:

**[00691]** 
$$\text{Total CD8 tumor-reactive yield} = (\text{Total Number of Cells Generated During the Pre-REP Stage (days 1-10)}) \times \text{Tumor-Specific Reactivity (A-B)} \times \text{Fold Expansion During the REP.}$$

**[00692]** The total number of CD8 tumor-reactive TILs generated from the TS-TIL process is on average 7-fold greater than the total number of CD8 tumor-reactive TILs generated from the CTRL process. See Fig. 50.

#### **EXAMPLE 18: SELECT CONDITIONS WHICH PERMIT MAXIMUM EXPANSION OF THE TUMORS-SPECIFIC CD8 TILS DURING THE REP STAGE**

**[00693]** To select conditions which permit maximum expansion of the tumors-specific CD8 TILs during the REP stage, multiple REP conditions were tested. The results for total viable cells and fold expansion are shown as a function of REP conditions. See Fig. 51. GEN2 (bulk TIL) control cells expanded much more robustly. However, among the 4-1BB and CD103 sorted populations, REP condition with IL-2 at 3000 IU/mL gave the best expansion of the tumor-specific TILs. By contrast, REP condition with Invigo-T (IL-15 + IL-21 both at 10 ng/mL and L-Arginine 5 mM) gave poor expansion.



**EXAMPLE 19: CD137 SORTING WITH HD IL-2 REP**

**[00694]** Fig. 52 shows the total yield of CD8+ tumor-reactive TILs (as assessed by autologous digest coculture ICS) as a function of sorting and REP conditions. CD137 (4-1BB) sorting followed by REP with high dose (HD) IL-2 (3000 ng/mL) was identified as the best process for expanded tumor specific TILs. Across the three patients (L4373, L4397, and L4375), TS-TIL yield improvements of 6.3, 23.7, and 4.7 fold were observed, respectively.

**[00695]** Viable CD8+ TILs were surface phenotyped using the markers CD45RA and CD62L, which broadly differentiate CD8+ T cells into four categories: CD45RA+CD62L+ (Tscm=stem cell memory), CD45RA-CD62+ (Tcm-central memory), CD45RA-CD62L- (Tem= effector memory), and CD45RA+CD62L- (Temra=terminal effector) cells. Cells were phenotyped during ICS staining as described previously. See Fig. 53. Based on the results, the majority (90-95%) of cells were found to be Tem “effector memory” cells, with minimal differences among the TILs made from different processes. By contrast only ~1% of CD8+ TILs were found be Tscm cells at the end of the REP.

**EXAMPLE 20: EXPANSION AND CHARACTERIZATION OF TILS WITH METABOLIC REPROGRAMING**

**[00696]** For this study, different strategies are investigated for REP TILs to enhance their expansion and phenotype. Literature suggested that some AA presence modulates T cell metabolism and enhances anti-tumor activity (Geiger et al., *Cell* **2016**, 167(3):829-842; Chang et al., *Cell* **2015**, 162(6):1229-41; the contents of which are hereby incorporated by reference in their entireties). Overall, this idea leads to reprogram metabolism in TILs for two major purposes: i) phenotypic and functional properties against tumor; and ii) survival and functionality in tumor microenvironment (TME) conditions. Indeed, arginine metabolic engineering in CAR-T cells to implement cell proliferation and therapeutic activity has been shown (Fultang et al., *Blood* **2020**, 136(10):1155-1160; the content of which is hereby incorporated by reference in its entirety).

**[00697]** **Materials and Methods**

**[00698]** T cells count and viability

**[00699]** REP cells were harvested after TIL expansion. Live cell numbers were assessed by AOPI assay using Cellometer cell counter machine (Nexcelom, Lawrence, MA).

[00700] Pre-REP and REP process

[00701] Human tumor samples were dissected into ~3 mm pieces and cultured in Grex10 with recommended culture medium containing IL-2 (6000IU/ml). After 11 days of culture, pre-REP cells were then propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and Invigo-T cocktail (10 ng/ml of IL-15 and 10 ng/ml of IL-21) for another 11 days.

[00702] Compounds to be incorporated during REP process:

L-Arginine: it has been optimized to 5mM. Since L-Arginine is a basic aa, it needs to be balanced with 25mM HEPES.

NAD<sup>+</sup>: curve for dose response has been performed.

GSK-3 $\alpha$ /b small molecule inhibitors (SMI).

[00703] Flow cytometry

[00704] To check T cell activation and differentiation, antibodies from BD Bioscience, Biolegend, ThermoFisher were used for phenotypic characterization. Data was acquired with a BioRad ZE5 flow cytometer and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR).

[00705] **Results**

[00706] L-Arginine enhances expansion and cell recovery post thaw during Invigo-T REP

[00707] First, the REP process was supplemented with different aa, and L-Arginine showed an increase in expansion and viability (Fig. 55A-B). Also, to mimic product standards prior to be delivered to a patient, REP's TILs were frozen and thawed to analyze recovery and killing capacity. L-Arginine protected the TILs for crashing (Fig. 55C-D).

[00708] L-Arginine provides better phenotype and killing capacity.

[00709] Supplementing L-Arginine during the REP showed some favorable phenotypic changes analyzed by flow cytometry, such as an increase in CD127, CD62L, CD25, CD28, ICOS and CD40L (Fig. 56A). Moreover, an increase in CD4<sup>+</sup>CD107<sup>+</sup> TILs was observed (Fig. 56B). So, the cytotoxicity of TILs was tested in an allogenic setting by coculturing TILs with KILR THP1 cells and doing multiple TxA stimulations in the REPs. REP'd TILs expanded with L-Arginine showed a better killing capacity (Fig. 56C).

[00710] NAD<sup>+</sup> synergizes with L-Arginine in boosting metabolism and providing a unique TIL signature.

[00711] TME conditions are challenging for TILs since nutrients are limited and conditions are not favorable. L-Arginine might act as a source of energy and helping the cells to enhance their functionality. In addition, to boost metabolism, the role of NAD<sup>+</sup> boosters were investigated during REP. Six different NAD<sup>+</sup> boosters (L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator) were screened, while NAD<sup>+</sup> showed an interesting phenotype in TIL product (data not shown). A dose-response with NAD<sup>+</sup> was performed and 50-75  $\mu$ M was found to be the best concentration to implement to the REP process (Fig. 57A-B) due to its minor impact on expansion but sustaining a favorable phenotype. In addition, NAD<sup>+</sup> supplement enhanced recovery of the cells from thawed samples, and even more prominent once the cells were activated with TransAct for 24h (Fig. 57C).

#### **EXAMPLE 21: LENTIVIRAL TRANSDUCTION EFFICIENCY AND PHENOTYPE OF TIL PRODUCTS PRODUCED USING VARIOUS IMPROVED REP CONDITIONS**

[00712] For this study, tumors were fragmented and cultured in CM2 containing IL-2 (6000 IU/mL) for 11 days. At the end of the 11 days, the cells (preREP TILs) were collected and either cryopreserved or processed fresh for further manufacturing. For cryopreserved preREP TILs, the cells were thawed and rested in CM2 containing IL-2 (300 IU/mL) for 3 days prior to further manufacturing. See Fig. 58.

[00713] For lentiviral transduction, 100,000 preREP TILs were transduced with a BaEVTR enveloped virus containing the nucleic acid sequence encoding for tethered IL-12 (TeIL-12) driven by an NFAT promoter, followed by a sequence encoding for truncated CD19 (tCD19) or tethered IL-15 (TeIL-15). See Fig. 76 for a vector map of pCMV-3Tag-4a-BaEV-TR (SEQ ID NO:165); Fig. 77 for a vector map of pLenti- IRES-NFAT-TeIL12-EF1a-TeIL-15 (SEQ ID NO:166). For spin-transduction, the TILs and virus were added to retronectin-coated plates in media containing IL-2 (3000 IU/mL), and then centrifuged for 45 min, 2000x g, 32°C. After spin-transduction, the TILs were incubated for 72 hrs prior to the initiation of REP. See Fig. 58. In parallel, preREP TILs were set cultured in the same conditions but did not undergo spin-transduction. These cells are referred to as Untransduced GEN2 Control.

[00714] After lentiviral transduction, TILs were placed into a REP in media containing OKT3 (30 ng/mL), irradiated allogenic feeder cells from 3 individual healthy donors and the following components:

1. GEN2: IL-2 (3000 IU/mL)
2. GEN2+NAD: IL-2 (3000 IU/mL) + NAD (50  $\mu$ M)
3. ITARG: IL-15 (10 ng/mL) + IL-21 (10 ng/mL) + L-arg (5 mM) + HEPES (25 mM)
4. ITARG+NAD: IL-15 (10 ng/mL) + IL-21 (10 ng/mL) + L-arg (5 mM) + HEPES (25 mM) + NAD (50  $\mu$ M)
5. TSREP: IL-2 (3000 IU/mL) + IL-21 (10 ng/mL) + L-arg (5 mM) + HEPES (25 mM) + NAD (50  $\mu$ M)

**[00715]** Five days after initiating the REP culture, a 50% media exchange was performed. The REP TILs were harvested 11 days after the initiation of the REP culture. See Fig. 58. The TILs in the REP product were then counted for total viable cells (TVC), cell viability, and phenotyped by flow cytometry.

**[00716]** Fig. 59 shows the transduction efficiency in CD3<sup>+</sup> T cells for NFAT-TeIL12-EF1 $\alpha$ -tCD19 (mean=30.3%) and NFAT-TeIL12-EF1 $\alpha$ -TeIL15 (mean= 23.2%). Fig. 60A shows the transduction efficiency in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets demonstrating that the expression of tCD19 in TILs transduced with NFAT-TeIL12-EF1 $\alpha$ -tCD19 is similar between CD4<sup>+</sup> and CD8<sup>+</sup> cells. In contrast, Fig. 60B shows the expression of TeIL15 in TILs transduced with NFAT-TeIL12-EF1 $\alpha$ -TeIL15 is higher in CD8<sup>+</sup> T cells compared to CD4<sup>+</sup> T cells.

**[00717]** Fig. 61 shows that the lentiviral vector transduced TILs exhibit low/minimal skewing of the percentages of CD4<sup>+</sup> or CD8<sup>+</sup> cells in the REP TIL product. Fig. 62 shows that the REP cell culture media conditions exhibit similar transduction efficiency in CD3<sup>+</sup> T cells. Fig. 63 shows that the REP cell culture media conditions exhibit similar transduction efficiency in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets.

**[00718]** Fig. 64 shows that the expansion rate and viability are enhanced with various REP cell culture media conditions. Fig. 65 shows that various REP cell culture media conditions increase the number of TILs (TVC) in untransduced TILs. Fig. 66 shows that various REP cell culture media conditions increase the viability in untransduced TILs.

**[00719]** The expression of TeIL-15 was measured on CD3<sup>+</sup> T cells and CD4<sup>+</sup>/CD8<sup>+</sup> T cell subsets. Results show that GEN2+NAD and ITARG+NAD REP conditions improve TeIL-15 expression in REP TILs (Fig. 67).

[00720] The TILs in the REP product were also cultured with autologous tumor digest cell suspensions. Untransduced TILs and NFAT-TeIL-12-EF1 $\alpha$  TeIL-15 TILs were thawed and cultured with autologous tumor digest at an Effector:Target ratio of 1:1 and incubated for 18-24 hours in CM2 media containing IL-2 (300IU/mL). TILs were cultured in the presence of tumor digest +/- anti-HLA-I (50 $\mu$ g/mL) and anti-HLA-II (10 $\mu$ g/mL) to measure HLA-dependent T cell activation. TILs were also cultured alone or stimulated with anti-CD3/anti-CD28 GMP TransAct (1:100 concentration). Supernatants were collected and analyzed for the concentration of IFN- $\gamma$ , TNF $\alpha$ , IL-12p70, IL-15 by the Bio-Plex Multiplex Immunoassay System (Bio-Rad).

[00721] Results show that IFN $\gamma$  and TNF $\alpha$  production is stimulated by autologous tumor digest (Fig. 68) and is HLA dependent (Fig. 69). This result demonstrates that the production of IFN $\gamma$  and TNF $\alpha$  is dependent upon the interaction of TILs with HLA molecules.

[00722] The results further show that NFAT-TeIL-12-EF1 $\alpha$ -TeIL-15 TILs produce more IFN $\gamma$  than untransduced TILs upon autologous stimulation which is enhanced by GEN2+NAD and ITARG+NAD (Fig. 70) and that NFAT-TeIL-12-EF1 $\alpha$ -TeIL-15 TILs produce more TNF $\alpha$  upon Autologous stimulation which is enhanced by GEN2+NAD, ITARG, and ITARG+NAD (Fig. 71).

#### **EXAMPLE 22: TILS TRANSDUCED WITH TEIL-12/TEIL-15 EXHIBIT SUPERIOR CYTOTOXICITY AGAINST AN AUTOLOGOUS MELANOMA CELL LINE**

[00723] For this study, melanoma tumor cells were grown in vitro after isolation from a patient derived xenograft. Melanoma cells were maintained in Medium 254 prior to culture with autologous TILs. First, 5000 melanoma cells were seeded on black-walled 96 well cell culture plates and incubated overnight in the Incucyte S3. Untransduced, NFAT-TeIL-12-EF1 $\alpha$ -tCD19, and NFAT-TeIL-12-EF1 $\alpha$ -TeIL-15 were added at various ET ratios, 8:1, 4:1, 2:1, 1:1, and 0.5:1 and incubated for 24hrs with a fluorescent Caspase3/7 dye to quantify tumor cell death.

[00724] TILs transduced with TeIL-12/TeIL-15 exhibit superior cytotoxicity against the autologous melanoma cell line (Fig. 72).

**EXAMPLE 23: TILS TRANSDUCED WITH TEIL-12/TEIL-15 EXHIBIT ENHANCED KILLING OF NY-ESO-1 A375 MELANOMA CELLS**

[00725] For this study, NYESO1 REP TIL were harvested and 5e6 cells of each condition were saved and incubated over the weekend in CM2 + 3000IU/mL IL-2 in a grex-24 well plate. A375 target cells were dissociated from their flasks with 3mL trypLE, washed with 27mL of CM2 media, centrifuged 4mins at 500g, and then resuspended at 2E5 cells/mL in TME media.

[00726] TME Media Preparation For 50mL of media: 3.125mL of DMEM + 10% FBS (High Glucose); 46.9mL of DMEM + 10% FBS (No Glucose); 30IU/mL IL2 and 0.09g Lactic Acid.

[00727] 50uL of TME media was added to each well of the Xcelligence plates and a background measurement was performed to allow for normalization across wells. 50uL of A375 target cells were then seeded on the Xcelligence plates at 1E4 cells/well in TME media. Cells were then incubated overnight in the Xcelligence incubator. On the following day NYESO1 REP TIL were harvested from the grex-24 well plate, counted and resuspended at , 5E4, 1.25E4 cells/mL and then 100uL of cells were added to wells in triplicate for each condition. Cells were cocultured for 48hrs according to the plate maps below and analyzed for killing function.

[00728] NYESO1 TCR-selected T-cells transduced with either the single NFAT-teIL-12 construct or the dual NFAT-teIL-12-EF1a-TeIL-15 construct show superior killing efficacy when compared to their lentivirus backbone control and mock process control (Fig. 73).

**EXAMPLE 24: OPTIMIZED MEDIA FORMULATION ENHANCES TIL EXPANSION AND CELL RECOVERY POST-THAW**

[00729] **Materials and Methods**

[00730] T cells count and viability

[00731] REP cells will be harvested after TIL expansion. Live cell numbers will be assessed by AOPI assay using Cellometer cell counter machine (Nexcelom, Lawrence, MA). REP TILs were cryofrozen using CS10 (StemCell Technologies, Vancouver, Canada) and

stored in liquid nitrogen until further use. Following post-thaw, Live cell numbers were assessed by AOPI assay using Cellometer cell counter machine.

[00732]        Pre-REP and REP process

[00733]        Human tumor samples were collected and dissected into ~3 mm pieces and cultured in Grex10 with recommended culture medium containing IL-2 (6000IU/ml). After 11 days of culture, pre-REP cells will be then propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and containing IL-2 (6000IU/ml). In parallel, preREP cells were propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and containing 10 ng/ml of IL-15 and 10 ng/ml of IL-21 together with 5mM of L-arginine (ITARG) without or with NAD<sup>+</sup> (ITARG+NAD<sup>+</sup>) for another 11 days.

[00734]        Gene-editing process

[00735]        To make TILs genetically modified to express tethered cytokines IL-12 and IL-15, 100,000 preREP TILs were transduced with a BaEVTR enveloped virus containing the nucleic acid sequence encoding for tethered IL-12 (TeIL-12) driven by an NFAT promoter, followed by a sequence encoding for tethered IL-15 (TeIL-15) driven by an EF1a promoter. For spin-transduction, the TILs and virus were added to retronectin-coated plates in media containing IL-2 (3000 IU/mL), and then centrifuged for 45 min, 2000x g, 32°C. After spin-transduction, the TILs were incubated for 72 hrs prior to the initiation of REP.

[00736]        Table 24 Summary of experimental conditions

| TIL                 | Pre-REP | REP                      |
|---------------------|---------|--------------------------|
| Unmodified TIL      | Gen2    | IL-2                     |
| Unmodified TIL      | Gen2    | IL-2 + NAD <sup>+</sup>  |
| Unmodified TIL      | Gen2    | ITARG                    |
| Unmodified TIL      | Gen2    | ITARG + NAD <sup>+</sup> |
| TeIL-12/TeIL-15 TIL | Gen2    | IL-2                     |
| TeIL-12/TeIL-15 TIL | Gen2    | IL-2 + NAD <sup>+</sup>  |
| TeIL-12/TeIL-15 TIL | Gen2    | ITARG                    |
| TeIL-12/TeIL-15 TIL | Gen2    | ITARG + NAD <sup>+</sup> |

[00737]        **Results**

[00738] First, the REP process was supplemented with IL-15 and IL-21 together with L-arginine (ITARG) without or with NAD<sup>+</sup> (ITARG+NAD<sup>+</sup>), resulting in an increase in expansion and viability of unmodified TIL compared with media formulation containing IL-2 only (Fig 74A-C). There was also a modest increase in the expansion of TIL genetically modified to express tethered cytokines IL-12 and IL-15 but a pronounced improvement in viability (Figure 75A-C). There was also an improvement of recovery of unmodified TIL post-thaw when expanded in conditions containing ITARG without or with NAD<sup>+</sup> (ITARG+NAD<sup>+</sup>) (Fig. 74D). In contrast, ITARG or ITARG+NAD<sup>+</sup> did not enhance the recovery of genetically modified TIL (Fig. 75D).

#### **EXAMPLE 25: OPTIMIZED MEDIA FORMULATION ENHANCES TIL EXPANSION, VIABILITY, AND CYTOTOXICITY**

##### **[00739] Materials and Methods**

##### **[00740] T cells count and viability**

[00741] REP cells were harvested after TIL expansion. Live cell numbers were assessed by AOPI assay using Cellometer cell counter machine (Nexcelom, Lawrence, MA). REP TILs were cryofrozen using CS10 (StemCell Technologies, Vancouver, Canada) and stored in liquid nitrogen until further use. Following post-thaw, live cell numbers were assessed by AOPI assay using Cellometer cell counter machine.

##### **[00742] Pre-REP and REP process**

[00743] Human tumor samples were collected and dissected into ~3 mm pieces and cultured in Grex10 with recommended culture medium containing IL-2 (6000IU/ml). After 11 days of culture, pre-REP cells were then propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and containing IL-2 (6000IU/ml). In parallel, preREP cells were propagated in a REP expansion process with irradiated PBMCs, anti-CD3 antibody and containing 10 ng/ml of IL-15 and 10 ng/ml of IL-21 together with 5mM of L-arginine (ITARG) without or with NAD<sup>+</sup> (ITARG+NAD<sup>+</sup>) for another 11 days.

##### **[00744] Gene-editing process**

[00745] To make TILs genetically modified to express tethered cytokines IL-12 and IL-15, 100,000 preREP TILs were transduced with a BaEVTR enveloped virus containing the nucleic acid sequence encoding for tethered IL-12 (TeIL-12) driven by an NFAT promoter, followed by a sequence encoding for tethered IL-15 (TeIL-15) driven by an EF1a promoter.



For spin-transduction, the TILs and virus were added to retronectin-coated plates in media containing IL-2 (3000 IU/mL), and then centrifuged for 45 min, 2000x g, 32°C. After spin-transduction, the TILs were incubated for 72 hrs prior to the initiation of REP.

**[00746] In vitro cytotoxicity assay**

**[00747]** Transgenic TCR targeting Kirsten rat sarcoma virus (KRAS) G12D mutation was used to evaluate the function of TIL without or with cytokine tethering in vitro. Patient-derived neoantigen-specific CD8 TCR recognizes the common KRAS G12D mutation presented by C\*08:02. HPAC pancreatic cancer cell line harbors the KRAS G12D mutation and expresses the C\*08:02 allele. Pre-REP TIL generated from tumor fragments were used for tumor-directed TIL production. CD8+ preREP TIL were transduced with either the KRAS lentivirus alone or co-transduced with the KRAS lentivirus and the tethered IL-12/IL-15 lentivirus (KRAS.mteth.IL12.IL15) by adding virus to 0.1e6 TIL in complete media supplemented with 3000 IU/mL IL-2 in a Retronectin coated 48 well non-tissue culture treated plate. The plate was centrifuged at 2000g for 45 minutes at 32°C, then placed in a 37°C incubator for 3 days. These cells were then sorted for CD8+TCRb+ expression before undergoing the REP process, as described above. To simulate conditions of the solid tumor microenvironment (TME), eGFP/mCherry-expressing HPAC cells were seeded in 96 well plates in a base media of DMEM (no glucose supplemented with 1.5 mM glucose, 20 mM lactic acid, 100 µM adenosine, 5 ng/mL TGFβ1 with or without 30 IU/mL IL-2 ) simulating the solid tumor microenvironment. Afterwards, KRAS TCR+ TIL or KRAS.mteth.IL12.IL15 TIL were added to HPAC cells in triplicate. A live-cell imaging system (Incucyte, Sartorius) was used to record TIL-based tumor-cell lysis at different effector-to-tumor (E:T) ratios over 4 days.

**[00748] Results**

**[00749] Optimized media formulation enhances TIL expansion and viability**

**[00750]** Media used during the REP process supplemented with IL-15 and IL-21 together with L-arginine (ITARG) without or with 50 µM NAD<sup>+</sup> (ITARG+NAD<sup>+</sup>) resulted in an increase in total viable cells (TVC), fold expansion and viability of unmodified TIL compared with media formulation containing IL-2 or IL-2 with 50 µM NAD<sup>+</sup> in 41 donors across various tumor types (Figs. 78A-C).

**[00751] Optimized media formulation enhances antigen-specific REP TIL cytotoxicity in vitro**

[00752] To evaluate the impact of media formulation on TIL function with respect to inhibiting tumor cell growth, pre-REP TILs transduced with a TCR construct targeting KRAS G12D were cultured with KRAS G12D-expressing HPAC tumor cells in conditions that simulate the solid TME. A modest benefit in controlling tumor growth was observed when antigen-specific TILs were expanded in optimized media formulations compared with media containing IL-2 only (IL-2/NAD<sup>+</sup>, 1.4 fold change; ITARG, 1.2 fold change; ITARG/NAD<sup>+</sup> 1.7 fold change) (Fig. 79A). Removing IL-2 (30 IU/mL) from the simulated TME resulted in a loss of TIL reactivity irrespective of media condition used to expand antigen-specific TIL (IL-2/NAD<sup>+</sup>, 1.05 fold change; ITARG, 1.03 fold change; ITARG/NAD<sup>+</sup> 1.2 fold change) (Fig. 79B).

[00753] **Membrane-tethered IL12/IL15 antigen-specific TILs show enhanced cytotoxicity in vitro**

[00754] Next, the impact of membrane-tethered cytokines on antigen-directed TIL expanded in optimized medias on tumor cell growth was evaluated upon coculture at low E:T ratios over time. Compared with all experimental groups, KRAS.mteth.IL15.IL21 TILs expanded in ITARG/NAD<sup>+</sup> exhibited the most potent antitumor activity (5.0 fold change) (Fig. 80A). Moreover, enhanced activity of KRAS.mteth.IL15.IL21 TILs expanded in ITARG/NAD<sup>+</sup> was maintained (5.2 fold change) in the absence of IL-2 (Fig. 80B). The level of mteth.IL15 on the surface of KRAS transduced TIL detected using flow cytometry serves as a control for transduction efficiency, as shown in Fig. 80C.

**EXAMPLE 26: MEMBRANE-TETHERED IL-12/IL-15 EXPRESSING TILS SHOW ENHANCED PERSISTENCE IN VIVO**

[00755] **Materials and Methods**

[00756] On Day -10, 1E6 PDX M1152 tumor cells were inoculated to 7 or 8 NOG mice for each experimental group #1-#7. On Day 0, 10E6 REP TILs were infused to each mouse in the treatment groups #2-#7 (see Table 25).

[00757] Table 25. PDX M1152 ACT experiment summary.

|                            | Group ID | Engineered REP-TIL | REP process | REP process |
|----------------------------|----------|--------------------|-------------|-------------|
| M1152 (ReREP-TIL) efficacy | 1        | M1152 tumor only   |             | 8           |
|                            | 2        | + ACT (Gen2 only)  | Gen2        | 7           |

|  |   |                                           |      |   |
|--|---|-------------------------------------------|------|---|
|  | 3 | + ACT (NFAT-TeIL-12/CD19)                 | Gen2 | 7 |
|  | 4 | +ACT( NFAT-TeIL-12/TeIL-15)               | Gen2 | 7 |
|  | 5 | + ACT (Gen2 only)<br>+Proleukin           | Gen2 | 7 |
|  | 6 | + ACT (NFAT-TeIL-12/CD19)<br>+Proleukin   | Gen2 | 7 |
|  | 7 | + ACT (NFAT-TeIL-12/TeIL15)<br>+Proleukin | Gen2 | 7 |

**[00758]** Nine doses of Proleukin (45,000IU) regimen were administered via ip injection for each mouse in treatment groups #5, #6 and #7; the administration schedule was:

Day 0: One dose Proleukin (45,000 IU) after ACT

Day 1: Two doses Proleukin (45,000 IU) - early morning and late afternoon

Day 2: Two doses Proleukin (45,000 IU) - early morning and late afternoon

Day 3: One dose Proleukin (45,000 IU)

Days 4-6: One dose Proleukin (45,000 IU) each day

**[00759]** Starting from Day 7, peripheral blood was collected from TIL-infused mice in EDTA-coated tubes as follows:

Day 7: Groups #2, #3, #4

Day 8: Groups #5, #6, #7

Day 14: Groups #2, #3, #4

Day 15: Groups #5, #6, #7

**[00760]** TILs persistence in PB were stained with CD2+CD3+ antibody and quantified by flow cytometry with absolute counting beads.

**[00761] Results**

**[00762]** TeIL-12/TeIL-15 expressing TILs show trends of increased persistence after infusion in the absence of Proleukin (Fig. 81).

\* \* \*

**[00763]** 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.

**[00764]** 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.

**[00765]** 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.

**[00766]** 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 nucleic acid molecule comprising a nucleotide sequence encoding a tethered IL-12 (TeIL-12), wherein the TeIL-12 is under the control of a nuclear factor of activated T-cells (NFAT)-responsive promoter.
2. The nucleic acid molecule of claim 1, wherein the TeIL-12 comprises a membrane anchor, and a human IL-12 p40 subunit fused to a human IL-12 p35 subunit.
3. The nucleic acid molecule of claim 2, wherein the human IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60 and the human IL-12 p40 subunit has the amino acid sequence of SEQ ID NO:61.
4. The nucleic acid molecule of any one of claims 1-3, wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.
5. The nucleic acid molecule of any one of claims 1-4, wherein the nucleotide sequence encoding the TeIL-12 is set forth in SEQ ID NO:162.
6. The nucleic acid molecule of any one of claims 1-5, further comprising a nucleotide sequence encoding a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, or a variant thereof.
7. The nucleic acid molecule of claim 6, wherein the cytokine is under the control of a constitutive promoter.
8. The nucleic acid molecule of claim 7, wherein the constitutive promoter is selected from the group consisting of an EF1 $\alpha$  promotor, a CMV promotor, a CAG promotor, an MND promotor, and an SSFV promotor.
9. The nucleic acid molecule of claim 7, wherein the constitutive promoter is an EF1 $\alpha$  promotor.
10. The nucleic acid molecule of any one of claims 6-9, wherein the cytokine is IL-15 or a variant thereof.
11. The nucleic acid molecule of claim 10, wherein the IL-15 is a human IL-15.
12. The nucleic acid molecule of claim 11, wherein the human IL-15 has the amino acid sequence of SEQ ID NO:64.
13. The nucleic acid molecule of any one of claims 10-12, wherein the IL-15 is a tethered IL-

- 15 (TeIL-15).
14. The nucleic acid molecule of claim 13, wherein the TeIL-15 comprises a membrane anchor, and a human IL-15.
15. The nucleic acid molecule of claim 14, wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.
16. The nucleic acid molecule of any one of claims 6-9, wherein the cytokine is IL-2 or a variant thereof.
17. The nucleic acid molecule of claim 16, wherein the IL-2 is a human IL-2.
18. The nucleic acid molecule of claim 17, wherein the human IL-2 has the amino acid sequence of SEQ ID NO:138.
19. The nucleic acid molecule of any one of claims 16-18, wherein the IL-2 is a tethered IL-2 (TeIL-2).
20. The nucleic acid molecule of claim 19, wherein the TeIL-2 has the amino acid sequence of SEQ ID NO:139.
21. The nucleic acid molecule of any one of claims 1-20, comprising the nucleic acid sequence set forth in SEQ ID NO:148-150.
22. The nucleic acid molecule of any one of claims 1-21, further comprising a nucleotide sequence encoding an shRNA.
23. The nucleic acid molecule of claim 22, wherein the shRNA inhibits the expression of an immune checkpoint gene.
24. The nucleic acid molecule of claim 23, wherein the immune checkpoint gene is selected from the group consisting of PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), CISH, TGF $\beta$ , 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.
25. The nucleic acid molecule of claim 23, wherein the immune checkpoint gene is PD-1.
26. The nucleic acid molecule of claim 25, wherein the PD-1 shRNA comprises a nucleic

acid sequence set forth in SEQ ID NO:141-147.

27. The nucleic acid molecule of any one of claims 1-26, comprising the nucleic acid sequence set forth in SEQ ID NO:151.
28. A recombinant expression vector comprising the nucleic acid molecule of any one of claims 1-27.
29. The recombinant expression vector of claim 28, wherein the vector is a transfer vector.
30. The recombinant expression vector of claim 29, wherein the transfer vector is derived from the human immunodeficiency virus-1 (HIV-1) and is replication deficient.
31. The recombinant expression vector of claim 30, wherein the transfer vector is a pLenti-IRES-EGFP vector.
32. A lentiviral expression system comprising the recombinant expression vector of claim 30 or 31 and one or more helper plasmids encoding an Env protein, a Gag protein, a Pol protein, and a Rev protein.
33. The lentiviral expression system of claim 32, comprising a helper plasmid encoding the Env protein, the Gag protein, the Pol protein, and the Rev protein.
34. The lentiviral expression system of claim 32, comprising a helper plasmid encoding the Env protein, and a helper plasmid encoding the Gag protein, the Pol protein, and the Rev protein.
35. The lentiviral expression system of claim 32, comprising a helper plasmid encoding the Env protein, the Gag protein, and the Pol protein, and a helper plasmid encoding the Rev protein.
36. The lentiviral expression system of claim 32, comprising a helper plasmid encoding the Env protein and the Rev protein, and a helper plasmid encoding the Gag protein and the Pol protein.
37. The lentiviral expression system of any one of claims 32-36, wherein the Env protein is selected from the group consisting of Ba-EVTR, VSV-G, and RD114.
38. The lentiviral expression system of claim 37, wherein the Env protein comprises a Ba-EVTR protein.
39. A method of making a recombinant lentiviral particle, comprising:

- a) culturing a population of packaging cells in a cell culture medium;
  - b) contacting the population of packaging cells with the lentiviral expression system of any one of claims 32-38; and
  - c) harvesting the supernatant of the cell culture medium,
- wherein the supernatant comprises the recombinant lentiviral particle.
- 40. A recombinant lentiviral RNA molecule comprising the nucleic acid molecule of any one of claims 1-27.
  - 41. A recombinant lentiviral proviral DNA comprising the nucleic acid molecule of any one of claims 1-27.
  - 42. A recombinant lentiviral particle comprising the recombinant lentiviral RNA molecule of claim 40.
  - 43. The recombinant lentiviral particle of claim 42, produced by the packaging cell line of any one of claims 240-284.
  - 44. The recombinant lentiviral particle of claim 42 or 43, comprising an Env protein selected from the group consisting of Ba-EVTR, VSV-G, and RD114.
  - 45. The recombinant lentiviral particle of any one of claims 42-44, further comprising a reverse transcriptase.
  - 46. The recombinant lentiviral particle of any one of claims 42-45, further comprising a capsid enclosing the recombinant lentiviral RNA molecule.
  - 47. A gene-edited tumor infiltrating lymphocyte (TIL) comprising the recombinant lentiviral proviral DNA of claim 41.
  - 48. The gene-edited TIL of claim 47, wherein the recombinant lentiviral proviral DNA is integrated into the genome of the gene-edited TIL.
  - 49. A gene-edited tumor infiltrating lymphocyte (TIL) expressing an exogenous tethered IL-12 (TeIL-12).
  - 50. The gene-edited TIL of claim 49, wherein the TeIL-12 comprises a membrane anchor, and a human IL-12 p40 subunit fused to a human IL-12 p35 subunit.
  - 51. The gene-edited TIL of claim 50, wherein the human IL-12 p35 subunit has the amino acid sequence of SEQ ID NO:60 and the human IL-12 p40 subunit has the amino acid



sequence of SEQ ID NO:61.

52. The gene-edited TIL of claim 51, wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.
53. The gene-edited TIL of any one of claims 49-52, further expressing a cytokine selected from the group consisting of IL-2, IL-6, IL-7, IL-9, IL-15, IL-18, IL-21, IL-23, IL-27, IL-33, IFN gamma, TNFa, IFN alpha, IFN beta, GM-CSF, GCSF, and a variant thereof.
54. The gene-edited TIL of claim 53, wherein the cytokine is IL-15 or a variant thereof.
55. The gene-edited TIL of claim 54, wherein the IL-15 is a human IL-15.
56. The gene-edited TIL of claim 55, wherein the human IL-15 has the amino acid sequence of SEQ ID NO:64.
57. The gene-edited TIL of any one of claims 54-56, wherein the IL-15 is a tethered IL-15 (TeIL-15).
58. The gene-edited TIL of claim 57, wherein the TeIL-15 comprises a membrane anchor, and a human IL-15.
59. The gene-edited TIL of claim 57, wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.
60. A method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising:
  - a) performing a first expansion by culturing a population of TILs in a first cell culture medium;
  - b) performing a second expansion by culturing the population of TILs in a second cell culture medium; and
  - c) at any time, transducing the population of TILs with the recombinant lentiviral particle of any one of claims 42-46 to produce the population of gene-edited TILs, wherein the population of gene-edited TILs expresses a TeIL-12.
61. The method of claim 60, further comprising activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle.
62. The method of claim 61, wherein the activating step comprises contacting the population of TILs with a cytokine selected from the group consisting of IL-2, IL-15, IL-21, IL-7,

and a combination thereof.

63. The method of claim 62, wherein the activating step comprises contacting the population of TILs with 20ng/mL IL-15.
64. The method of claim 62, wherein the activating step comprises contacting the population of TILs with 10 ng/mL IL-7.
65. The method of claim 62, wherein the activating step comprises contacting the population of TILs with TransAct.
66. The method of claim 62, wherein the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100.
67. The method of any one of claims 60-66, wherein the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL.
68. The method of any one of claims 60-67, wherein the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40.
69. The method of any one of claims 60-68, wherein the transducing step is conducted in the presence of RetroNectin or Vectofusin-1.
70. The method of any one of claims 60-69, wherein the transducing step comprises centrifugation.
71. The method of any one of claims 60-70, wherein the transducing step is conducted in the presence of Lentibooster.
72. The method of any one of claims 60-71, wherein the transducing step is conducted after the first expansion step and before the second expansion step.
73. The method of any one of claims 61-72, wherein the activating step is conducted after the first expansion step and before the second expansion step.
74. The method of any one of claims 60-73, further comprising resting the population of TILs for 2 day or 3 days after the transducing step.
75. The method of any one of claims 60-74, wherein the population of TILs is obtained from a tumor digest.
76. The method of any one of claims 60-75, further comprising a priming step.
77. The method of claim 76, wherein the priming step is performed before the first expansion

step.

78. The method of claim 76 or 77, wherein the priming step is performed in the presence of IFN $\gamma$ .
79. The method of claim 78, wherein IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step.
80. The method of claim any one of claims 76-79, wherein the priming step is performed in the presence of IL-2, IL-15, and/or IL-21.
81. The method of claim 80, wherein the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step.
82. The method of claim 80 or 81, wherein the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step.
83. The method of claim 80 or 81, wherein the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step.
84. The method of any one of claims 76-83, wherein the priming step lasts for 24-48 hours.
85. The method of any one of claims 76-83, wherein the priming step lasts for about 30 hours.
86. The method of any one of claims 60-85, further comprising an enrichment step.
87. The method of claim 86, wherein the enrichment step is performed after the priming step and before the first expansion step.
88. The method of claim 86 or 87, wherein the population of TILs is enriched for CD137+ T cells.
89. The method of claim 86 or 87, wherein the population of TILs is enriched for CD137+ and CD39+ T cells.
90. The method of claim 86 or 87, wherein the population of TILs is enriched for CD137+ and CD200+ T cells.
91. The method of claim 86 or 87, wherein the population of TILs is enriched for CD137+, CD200+, CD39+, and/or OX40+ T cells.
92. The method of any one of claims 60-91, wherein the first expansion is conducted in the presence of feeder cells.

93. The method of claim 92, wherein the feeder cells are T cell-depleted PBMCs.
94. The method of any one of claims 60-93, wherein the first expansion is performed over a period of about 3-11 days.
95. The method of any one of claims 60-93, wherein the first expansion is performed over a period of about 9 days.
96. The method of any one of claims 60-95, wherein the second cell culture medium comprises IL-15 and IL-21.
97. The method of claim 96, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
98. The method of any one of claims 60-97, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine.
99. The method of claim 98, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.
100. The method of claim 98, wherein the L-arginine is present at a concentration of about 5 mM.
101. The method of any one of claims 60-100, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
102. The method of claim 101, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
103. The method of any one of claims 60-102, wherein the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ .
104. The method of claim 103, wherein the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.
105. The method of any one of claims 60-104, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and a protein kinase B (AKT) inhibitor.
106. The method of claim 105, wherein the AKT inhibitor is selected from the group consisting of ipatasertib, GSK690693, GSK2141795, GSK2110183, AZD5363, GDC-0068, AT7867, CCT128930, MK-2206, BAY 1125976, Perifosine, Oridonin, Herbacetin,

Tehranolide, Isoliquiritigenin, Scutellarin, and Honokiol.

107. The method of claim 105, wherein the AKT inhibitor is AZD5363.
108. The method of any one of claims 105-107, wherein the AKT inhibitor is at a concentration of about 0.1  $\mu$ M to about 10  $\mu$ M.
109. The method of any one of claims 105-107, wherein the AKT inhibitor is at a concentration of about 1  $\mu$ M.
110. The method of any one of claims 60-109, wherein the second expansion is performed over a period of about 7-11 days.
111. The method of any one of claims 60-109, wherein the second expansion is performed over a period of about 10 days.
112. A method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising:
  - a) priming a tumor digest comprising a population of TILs in the presence of IFN $\gamma$ ;
  - b) enriching the population of TILs for CD137+ T cells;
  - c) performing a first expansion by culturing the population of TILs enriched for CD137+ T cells in a first cell culture medium;
  - d) transducing the population of TILs enriched for CD137+ T cells with a recombinant lentiviral particle comprising a nucleic acid sequence encoding TeIL-12 to produce the population of gene-edited TILs; and
  - e) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium.
113. The method of claim 112, wherein the TeIL-12 is under the control of an NFAT promoter.
114. The method of claim 112 or 113, wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.
115. The method of claim 112 or 113, wherein the nucleotide sequence encoding the TeIL-12 is set forth in SEQ ID NO:162.
116. The method of any one of claims 112-115, further comprising activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the

recombinant lentiviral particle.

117. The method of claim 116, wherein the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100.
118. The method of any one of claims 112-117, wherein the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL.
119. The method of any one of claims 112-118, wherein the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40.
120. The method of any one of claims 112-119, wherein the transducing step is conducted in the presence of RetroNectin or Vectofusin-1.
121. The method of any one of claims 112-120, wherein the transducing step comprises centrifugation.
122. The method of any one of claims 112-121, wherein the transducing step is conducted in the presence of Lentibooster.
123. The method of any one of claims 112-122, further comprising resting the population of TILs for 2 day or 3 days after the transducing step.
124. The method of any one of claims 112-123, wherein IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step.
125. The method of any one of claims 112-124, wherein the priming step is performed in the presence of IL-2, IL-15, and/or IL-21.
126. The method of claim 125, wherein the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step.
127. The method of claim 125 or 126, wherein the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step.
128. The method of any one of claims 112-127, wherein the priming step lasts for about 30 hours.
129. The method of any one of claims 112-128, wherein the first expansion is conducted in the presence of feeder cells.
130. The method of claim 129, wherein the feeder cells are T cell-depleted PBMCs.
131. The method of any one of claims 112-130, wherein the first expansion is performed in

the presence of IL-2, IL-15, and/or IL-21.

132. The method of claim 131, wherein the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step.
133. The method of claim 130 or 131, wherein the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the first expansion.
134. The method of any one of claims 112-133, wherein the first expansion is performed over a period of about 9 days.
135. The method of any one of claims 112-134, wherein the second cell culture medium comprises IL-15 and IL-21.
136. The method of claim 135, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
137. The method of any one of claims 112-136, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine.
138. The method of claim 137, wherein the L-arginine is present at a concentration of about 5 mM.
139. The method of any one of claims 112-138, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
140. The method of claim 139, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
141. The method of any one of claims 112-140, wherein the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ .
142. The method of claim 141, wherein the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.
143. The method of any one of claims 112-142, wherein the second expansion is performed over a period of about 10 days.
144. A method of making a population of gene-edited tumor-infiltrating lymphocytes (TILs), comprising:
- a) priming a tumor digest comprising a population of TILs in the presence of IFN $\gamma$ ;

- b) enriching the population of TILs for CD137+ T cells;
  - c) performing a first expansion by culturing the population of TILs enriched for CD137+ T cells in a first cell culture medium;
  - d) transducing the population of TILs enriched for CD137+ T cells with a recombinant lentiviral particle comprising a nucleic acid sequence encoding TeIL-12 and TeIL-15 to produce the population of gene-edited TILs; and
  - e) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium.
145. The method of claim 144, wherein the TeIL-12 is under the control of an NFAT promoter.
146. The method of claim 144 or 145, wherein the TeIL-15 is under the control of a constitutive promoter.
147. The method of claim 146, wherein the constitutive promoter is an EF1 $\alpha$  promoter.
148. The method of any one of claims 145-147, wherein the NFAT promoter and the constitutive promoter are introduced in the same nucleic acid of the coding sequence.
149. The method of any one of claims 144-148, wherein the TeIL-12 comprises an amino acid sequence set forth in SEQ ID NO:62.
150. The method of any one of claims 144-148, wherein the nucleotide sequence encoding the TeIL-12 is set forth in SEQ ID NO:162.
151. The method of any one of claims 144-150, wherein the TeIL-15 has the amino acid sequence of SEQ ID NO:73.
152. The method of any one of claims 144-151, further comprising activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle.
153. The method of claim 152, wherein the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100.
154. The method of any one of claims 144-153, further comprising resting the population of TILs for 2 day or 3 days after the transducing step.
155. The method of any one of claims 144-154, wherein IFN $\gamma$  is present at a concentration



of 200 ng/mL during the priming step.

156. The method of any one of claims 144-155, wherein the priming step is performed in the presence of IL-2, IL-15, and/or IL-21.
157. The method of claim 156, wherein the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step.
158. The method of claim 156 or 157, wherein the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step.
159. The method of any one of claims 144-158, wherein the priming step lasts for about 30 hours.
160. The method of any one of claims 144-159, wherein the first expansion is conducted in the presence of feeder cells.
161. The method of claim 160, wherein the feeder cells are T cell-depleted PBMCs.
162. The method of any one of claims 144-161, wherein the first expansion is performed over a period of about 9 days.
163. The method of any one of claims 144-162, wherein the second cell culture medium comprises IL-15 and IL-21.
164. The method of claim 163, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
165. The method of any one of claims 144-164, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine.
166. The method of claim 165, wherein the L-arginine is present at a concentration of about 5 mM.
167. The method of any one of claims 144-166, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
168. The method of claim 167, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
169. The method of any one of claims 144-168, wherein the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ .
170. The method of claim 169, wherein the inhibitor of GSK-3 $\alpha/\beta$  is selected from the

group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.

171. The method of any one of claims 144-170, wherein the second expansion is performed over a period of about 10 days.
172. A therapeutic population of gene-edited TILs produced by the method of any one of claims 60-171.
173. The therapeutic population of gene-edited TILs of claim 172, wherein about 15-60% of the population of gene-edited TILs expresses the TeIL-12.
174. The therapeutic population of gene-edited TILs of claim 172, wherein more than 30% of the population of gene-edited TILs expresses the TeIL-12.
175. The therapeutic population of gene-edited TILs of claim 172, wherein more than 50% of the population of gene-edited TILs expresses the TeIL-12.
176. The therapeutic population of gene-edited TILs of any one of claims 172-175, wherein about 15-60% of the population of gene-edited TILs expresses the TeIL-15.
177. The therapeutic population of gene-edited TILs of any one of claims 172-175, wherein more than 30% of the population of gene-edited TILs expresses the TeIL-15.
178. The therapeutic population of gene-edited TILs of any one of claims 172-175, wherein more than 50% of the population of gene-edited TILs expresses the TeIL-15.
179. The therapeutic population of gene-edited TILs of any one of claims 172-178, wherein about 15-60% of the population of gene-edited TILs expresses the TeIL-2.
180. The therapeutic population of gene-edited TILs of any one of claims 172-178, wherein more than 30% of the population of gene-edited TILs expresses the TeIL-2.
181. The therapeutic population of gene-edited TILs of any one of claims 172-178, wherein more than 50% of the population of gene-edited TILs expresses the TeIL-2.
182. The therapeutic population of gene-edited TILs of any one of claims 172-181, wherein more than 30% of the population of gene-edited TILs has reduced expression of PD-1.
183. The therapeutic population of gene-edited TILs of any one of claims 172-181, wherein more than 40% of the population of gene-edited TILs has reduced expression of PD-1.

184. The therapeutic population of gene-edited TILs of any one of claims 172-181, wherein more than 60% of the population of gene-edited TILs has reduced expression of PD-1.
185. A pharmaceutical composition comprising the therapeutic population of gene-edited TILs of any one of claims 172-184.
186. A method of treating a cancer in a patient in need thereof comprising:
- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
  - b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
  - c) performing a second expansion by culturing the population of TILs in a second cell culture medium;
  - d) at any time, transducing the population of TILs with the recombinant lentiviral particle of any one of claims 42-46 to produce a therapeutic population of gene-edited TILs; and
  - e) administering the therapeutic population of gene-edited TILs to the patient.
187. The method of claim 186, further comprising activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle.
188. The method of claim 187, wherein the activating step comprises contacting the population of TILs with a cytokine selected from the group consisting of IL-2, IL-15, IL-21, IL-7, and a combination thereof.
189. The method of claim 187, wherein the activating step comprises contacting the population of TILs with TransAct.
190. The method of any one of claims 186-189, wherein the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL.
191. The method of any one of claims 186-190, wherein the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40.
192. The method of any one of claims 186-191, wherein the transducing step is conducted in the presence of RetroNectin or Vectofusin-1.

193. The method of any one of claims 186-192, wherein the transducing step comprises centrifugation.
194. The method of any one of claims 186-193, wherein the transducing step is conducted in the presence of Lentibooster.
195. The method of any one of claims 186-194, wherein the transducing step is conducted after the first expansion step and before the second expansion step.
196. The method of any one of claims 187-195, wherein the activating step is conducted after the first expansion step and before the second expansion step.
197. The method of any one of claims 186-196, further comprising resting the population of TILs for 2 day or 3 days after the transducing step.
198. The method of any one of claims 186-197, wherein the population of TILs is obtained from a tumor digest.
199. The method of claim 198, further comprising a priming step, wherein the tumor digest is primed in the presence of IL-2, IL-15, IL-21, and IFN $\gamma$ .
200. The method of claim 199, wherein IFN $\gamma$  is present at a concentration of 200 ng/mL during the priming step.
201. The method of claim 199, wherein the IL-2 is at a concentration of 3000 IU/mL or lower during the priming step.
202. The method of any one of claims 199-201, wherein the IL-15 and/or IL-21 is present at a concentration of about 1 ng/mL to about 100 ng/mL during the priming step.
203. The method of any one of claims 199-201, wherein the IL-15 and/or IL-21 is present at a concentration of about 10 ng/mL during the priming step.
204. The method of any one of claims 199-203, wherein the priming step lasts for 24-48 hours.
205. The method of any one of claims 199-203, wherein the priming step lasts for about 30 hours.
206. The method of any one of claims 186-205, further comprising an enrichment step, wherein the population of TILs is enriched for CD137+ T cells.
207. The method of any one of claims 186-205, further comprising an enrichment step,

- wherein the population of TILs is enriched for CD137+ and CD39+T cells.
208. The method of any one of claims 186-205, further comprising an enrichment step, wherein the population of TILs is enriched for CD137+ and CD200+T cells.
209. The method of any one of claims 186-205, further comprising an enrichment step, wherein the population of TILs is enriched for CD137+, CD200+, CD39+, and/or OX40+ T cells.
210. The method of any one of claims 186-209, wherein the first expansion is conducted in the presence of feeder cells.
211. The method of claim 210, wherein the feeder cells are T cell-depleted PBMCs.
212. The method of any one of claims 186-211, wherein the first expansion is performed over a period of about 3-11 days.
213. The method of any one of claims 186-211, wherein the first expansion is performed over a period of about 9 days.
214. The method of any one of claims 186-213, wherein the second cell culture medium comprises IL-15 and IL-21.
215. The method of claim 214, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
216. The method of any one of claims 186-215, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine.
217. The method of claim 216, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.
218. The method of claim 216, wherein the L-arginine is present at a concentration of about 5 mM.
219. The method of any one of claims 186-218, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
220. The method of claim 219, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
221. The method of any one of claims 186-220, wherein the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ .

222. The method of claim 221, wherein the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.
223. The method of any one of claims 186-222, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and a protein kinase B (AKT) inhibitor.
224. The method of claim 223, wherein the AKT inhibitor is selected from the group consisting of ipatasertib, GSK690693, GSK2141795, GSK2110183, AZD5363, GDC-0068, AT7867, CCT128930, MK-2206, BAY 1125976, Perifosine, Oridonin, Herbacetin, Tehranolide, Isoliquiritigenin, Scutellarin, and Honokiol.
225. The method of claim 223, wherein the AKT inhibitor is AZD5363.
226. The method of any one of claims 223-225, wherein the AKT inhibitor is at a concentration of about 0.1  $\mu$ M to about 10  $\mu$ M.
227. The method of any one of claims 223-225, wherein the AKT inhibitor is at a concentration of about 1  $\mu$ M.
228. The method of any one of claims 186-227, wherein the second expansion is performed over a period of about 7-11 days.
229. The method of any one of claims 186-227, wherein the second expansion is performed over a period of about 10 days.
230. The method of any one of claims 186-227, wherein the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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.
231. The method of any one of claims 186-230, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient.
232. The method of claim 231, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for

two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.

233. The method of claim 231, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.
234. The method of claim 231, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day.
235. The method of any one of claims 232-234, wherein the cyclophosphamide is administered with mesna.
236. The method of any one of claims 186-235, further comprising a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient.
237. The method of any one of claims 186-235, further comprising a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient.
238. The method of claim 236 or 237, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.
239. The method of any one of claims 186-235, comprising no step of treating the patient with an IL-2 regimen.
240. A packaging cell line comprising the recombinant expression vector of any one of claims 28-31, and one or more recombinant DNA molecules, each of which one or more recombinant DNA molecules encodes any one protein, any two proteins, any three proteins or all four proteins selected from the group consisting of encoding an Env protein, a Gag protein, a Pol protein, and a Rev protein, with the proviso that any protein selected from the group consisting of the Env protein, the Gag protein, the Pol protein and the Rev protein is encoded by at least one recombinant DNA molecule of the one or more

recombinant DNA molecules, wherein each of the one or more recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid.

241. The packaging cell line of claim 240, wherein the one or more recombinant DNA molecules consist of a first recombinant DNA molecule.
242. The packaging cell line of claim 241, wherein the first recombinant DNA molecule is either integrated into the genome of the packaging cell line or comprised by a helper plasmid.
243. The packaging cell line of claim 241, wherein the one or more recombinant DNA molecules consist of a first recombinant DNA molecule and a second recombinant DNA molecule.
244. The packaging cell line of claim 243, wherein the first recombinant DNA molecule encodes a single protein selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein.
245. The packaging cell line of claim 244, wherein the first recombinant DNA molecule encodes the Env protein.
246. The packaging cell line of claim 244, wherein the first recombinant DNA molecule encodes the Gag protein.
247. The packaging cell line of claim 244, wherein the first recombinant DNA molecule encodes the Pol protein.
248. The packaging cell line of claim 244, wherein the first recombinant DNA molecule encodes the Rev protein.
249. The packaging cell line of claim 243, wherein the first recombinant DNA molecule encodes two proteins selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein.
250. The packaging cell line of claim 249, wherein the first recombinant DNA molecule encodes the Env protein and the Gag protein.
251. The packaging cell line of claim 249, wherein the first recombinant DNA molecule encodes the Env protein and the Pol protein.
252. The packaging cell line of claim 249, wherein the first recombinant DNA molecule



encodes the Env protein and the Rev protein.

253. The packaging cell line of claim 249, wherein the first recombinant DNA molecule encodes the Gag protein and the Pol protein.
254. The packaging cell line of claim 249, wherein the first recombinant DNA molecule encodes the Gag protein and the Rev protein.
255. The packaging cell line of claim 249, wherein the first recombinant DNA molecule encodes the Pol protein and the Rev protein.
256. The packaging cell line of any one of claims 243-255, wherein each of the first and second recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid.
257. The packaging cell line of claim 240, wherein the one or more recombinant DNA molecules consist of a first recombinant DNA molecule, a second recombinant DNA molecule, and a third recombinant DNA molecule.
258. The packaging cell line of claim 257, wherein the first recombinant DNA molecule encodes a first protein and the second recombinant DNA molecule encodes a second protein, wherein the first and second proteins are independently selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein, with the proviso that the first and second proteins are not the same.
259. The packaging cell line of claim 258, wherein the first protein is the Env protein and the second protein is the Gag protein.
260. The packaging cell line of claim 258, wherein the first protein is the Env protein and the second protein is the Pol protein.
261. The packaging cell line of claim 258, wherein the first protein is the Env protein and the second protein is the Rev protein.
262. The packaging cell line of claim 258, wherein the first protein is the Gag protein and the second protein is the Pol protein.
263. The packaging cell line of claim 258, wherein the first protein is the Gag protein and the second protein is the Rev protein.
264. The packaging cell line of claim 258, wherein the first protein is the Pol protein and the second protein is the Rev protein.

265. The packaging cell line of claim 257, wherein the first recombinant DNA molecule encodes a first protein and the second recombinant DNA molecule encodes two proteins, wherein the first protein and each of the two proteins are independently selected from the group consisting of the Env protein, the Gag protein, the Pol protein, and the Rev protein, with the proviso that any protein is not the same as any other protein in the group consisting of the first protein and the two proteins.
266. The packaging cell line of claim 265, wherein the first protein is the Env protein, and the two proteins are the Gag protein and the Pol protein.
267. The packaging cell line of claim 265, wherein the first protein is the Env protein, and the two proteins are the Gag protein and the Rev protein.
268. The packaging cell line of claim 265, wherein the first protein is the Env protein, and the two proteins are the Pol protein and the Rev protein.
269. The packaging cell line of claim 265, wherein the first protein is the Gag protein, and the two proteins are the Env protein and the Pol protein.
270. The packaging cell line of claim 265, wherein the first protein is the Gag protein, and the two proteins are the Env protein and the Rev protein.
271. The packaging cell line of claim 265, wherein the first protein is the Gag protein, and the two proteins are the Pol protein and the Rev protein.
272. The packaging cell line of claim 265, wherein the first protein is the Pol protein, and the two proteins are the Env protein and the Gag protein.
273. The packaging cell line of claim 265, wherein the first protein is the Pol protein, and the two proteins are the Env protein and the Rev protein.
274. The packaging cell line of claim 265, wherein the first protein is the Pol protein, and the two proteins are the Gag protein and the Rev protein.
275. The packaging cell line of claim 265, wherein the first protein is the Rev protein, and the two proteins are the Env protein and the Gag protein.
276. The packaging cell line of claim 265, wherein the first protein is the Rev protein, and the two proteins are the Env protein and the Pol protein.
277. The packaging cell line of claim 265, wherein the first protein is the Rev protein, and the two proteins are the Gag protein and the Pol protein.

278. The packaging cell line of any one of claims 257-277, wherein each of the first, second, and third recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid .
279. The packaging cell line of claim 240, wherein the one or more recombinant DNA molecules consist of a first recombinant DNA molecule, a second recombinant DNA molecule, a third recombinant DNA molecule, and a fourth recombinant DNA molecule.
280. The packaging cell line of claim 279, wherein each of the first, second, third, and fourth recombinant DNA molecules is either integrated into the genome of the packaging cell line or comprised by a helper plasmid.
281. The packaging cell line of any one of claims 240-280, wherein the Env protein comprises a Ba-EVTR protein.
282. The packaging cell line of any one of claims 240-281, wherein the nucleic acid molecule encoding TeIL-12 is integrated into the genome of the packaging cell line.
283. The packaging cell line of any one of claims 240-282, wherein the recombinant DNA molecule encoding one or more the Env protein, the Gag protein, the Pol protein, and the Rev protein is under the control of an inducible promoter.
284. The packaging cell line of any one of claims 240-283, comprising a 293T cell line.
285. A method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising:
- a. priming a population of TILs obtained from a tumor digest in the presence of IFN $\gamma$ ;
  - b. enriching the population of TILs for CD137+ TILs;
  - c. performing a first expansion by culturing the population of TILs enriched for CD137+ T cells in a first cell culture medium; and
  - d. performing a second expansion by culturing the population of TILs enriched for CD137+ TILs in a second cell culture medium to produce a population of TILs enriched for tumor-reactive TILs.
286. The method of claim 285, wherein the population of TILs enriched for tumor-reactive TILs comprises an increased percentage of tumor-reactive TILs in comparison to TILs expanded using a reference expansion procedure.

287. The method of claim 285 or 286, wherein step (a) is performed for about 30 hours.
288. The method of any one of claims 285-287, wherein step (c) is performed for about 9 days.
289. The method of any one of claims 285-288, wherein step (d) is performed for about 10 days.
290. The method of any one of claims 285 -289, wherein the first cell culture medium comprises IL-2 at a concentration of 3000 IU/mL, IL-15 at a concentration of 10 ng/mL, and IL-21 at a concentration of 10 ng/mL.
291. The method of any one of claims 285-289, wherein the first cell culture medium comprises IL-2 at a concentration of 6000 IU/mL.
292. The method of any one of claims 285-291, wherein step (c) is performed in the presence of iPBMC.
293. The method of claim 292, wherein the iPBMC is depleted of T cells.
294. The method of any one of claims 285-293, wherein the IFN $\gamma$  is present at a concentration of 200 ng/mL.
295. The method of any one of claims 285-294, wherein the second cell culture medium comprises IL-2 at a concentration of 3000 IU/mL, IL-21 at a concentration of 10 ng/mL, and IL-15 at a concentration of 10 ng/mL.
296. The method of any one of claims 285-295, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine.
297. The method of claim 296, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.
298. The method of claim 296, wherein the L-arginine is present at a concentration of about 5 mM.
299. The method of any one of claims 285-298, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
300. The method of claim 299, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
301. The method of any one of claims 285-300, wherein the second cell culture medium

comprises an inhibitor of GSK-3 $\alpha/\beta$ .

302. The method of claim 301, wherein the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.
303. The method of any one of claims 285-302, wherein the enriching the population of TILs for CD137+ TILs comprises contacting the population of TILs with anti-CD137 antibody immobilized on a bead.
304. The method of any one of claims 285-303, further comprising contacting the population of TILs with an anti-CD39 antibody.
305. The method of any one of claims 285-304, further comprising contacting the population of TILs with an anti-CD200 antibody.
306. A method for enriching tumor-reactive tumor infiltrating lymphocytes (TILs), comprising:
- a. priming a population of TILs obtained from a tumor digest in the presence of IFN $\gamma$ ;
  - b. enriching the population of TILs for CD103+ TILs;
  - c. performing a first expansion by culturing the population of TILs enriched for CD103+ T cells in a first cell culture medium; and
  - d. performing a second expansion by culturing the population of TILs enriched for CD103+ T cells in a second cell culture medium to produce a population of TILs enriched for tumor-reactive TILs.
307. The method of claim 306, wherein the population of TILs enriched for tumor-reactive TILs comprises an increased percentage of tumor-reactive TILs in comparison to TILs expanded using a reference expansion procedure.
308. The method of claim 306 or 307, wherein step (a) is performed for about 24 hours.
309. The method of any one of claims 306-308, wherein selecting CD103+ TILs comprises sorting the second population of TILs using flow cytometry.
310. The method of any one of claims 306-309, wherein step (b) comprises selecting CD103+CD31- TILs.

311. The method of any one of claims 306-310, wherein step (c) is performed for about 9 days.
312. The method of any one of claims 306-311, wherein step (d) is performed for about 10 days.
313. The method of any one of claims 306-312, wherein the first cell culture medium comprises IL-2 at a concentration of 3000 IU/mL, IL-15 at a concentration of 10 ng/mL, and IL-21 at a concentration of 10 ng/mL.
314. The method of any one of claims 306-313, wherein the first cell culture medium comprises IL-2 at a concentration of 6000 IU/mL.
315. The method of any one of claims 306-314, wherein step (c) is performed in the presence of iPBMC.
316. The method of claim 315, wherein the iPBMC is depleted of T cells.
317. The method of any one of claims 306-316, wherein the IFN $\gamma$  is present at a concentration of 200 ng/mL.
318. The method of any one of claims 306-317, wherein the second cell culture medium comprises IL-2 at a concentration of 3000 IU/mL, IL-21 at a concentration of 10 ng/mL, and IL-15 at a concentration of 10 ng/mL.
319. The method of any one of claims 306-318, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), and L-arginine.
320. The method of claim 319, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.
321. The method of claim 319, wherein the L-arginine is present at a concentration of about 5 mM.
322. The method of any one of claims 306-321, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
323. The method of claim 322, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
324. The method of any one of claims 306-323, wherein the second cell culture medium comprises an inhibitor of GSK-3 $\alpha/\beta$ .

325. The method of claim 324, wherein the inhibitor of GSK-3 $\alpha/\beta$  is selected from the group consisting of SB415286, SB216763, CHIR99021, AR-AO14418, TZD8, TWS119, Lithium Chloride hydrate, BIO, and 3F8.
326. A method of treating a cancer in a patient in need thereof comprising:
- resecting a tumor sample from the patient;
  - digesting the tumor sample to obtain a tumor digest;
  - producing a population of TILs enriched for tumor-reactive TILs from the tumor digest using the method of any one of claims 285-325; and
  - administering the therapeutic population of gene-edited TILs to the patient.
327. The method of claim 326, wherein the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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.
328. The method of claim 326 or 327, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient.
329. The method of claim 328, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.
330. The method of claim 328, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.
331. The method of claim 328, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day.

332. The method of any one of claims 329-331, wherein the cyclophosphamide is administered with mesna.
333. The method of any one of claims 326-332, further comprising a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient.
334. The method of any one of claims 326-332, further comprising a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient.
335. The method of claim 333 or 334, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.
336. The method of any one of claims 326-332, comprising no step of treating the patient with an IL-2 regimen.
337. A method of making a population of tumor-infiltrating lymphocytes (TILs), comprising:
- a) performing a first expansion by culturing a population of TILs in a first cell culture medium; and
  - b) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, and L-arginine.
338. The method of claim 337, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
339. The method of claim 337 or 338, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.
340. The method of claim 337 or 338, wherein the L-arginine is present at a concentration of about 5 mM.
341. The method of any one of claims 337-340, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
342. The method of claim 341, wherein the NAD<sup>+</sup> booster is selected from the group



consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.

343. The method of claim 341, wherein the NAD<sup>+</sup> booster is NAD<sup>+</sup>.
344. The method of claim 343, wherein the NAD<sup>+</sup> is present at a concentration of about 50-75  $\mu$ M.
345. The method of any one of claims 337-344, wherein the first expansion is performed over a period of about 3-14 days.
346. The method of any one of claims 337-344, wherein the first expansion is performed over a period of about 11 days.
347. The method of any one of claims 337-345, wherein the second expansion is performed over a period of about 7-14 days.
348. The method of any one of claims 337-345, wherein the second expansion is performed over a period of about 11 days.
349. A therapeutic population of TILs produced by the method of any one of claims 337-348.
350. A pharmaceutical composition comprising the therapeutic population of TILs of claim 349.
351. A method of treating a cancer in a patient in need thereof comprising:
- a) resecting a tumor sample from the patient, wherein the tumor sample comprises a population of TILs;
  - b) performing a first expansion by culturing the population of TILs in a first cell culture medium;
  - c) performing a second expansion by culturing the population of TILs in a second cell culture medium, wherein the second cell culture medium comprises OKT-3, antigen presenting cells (APCs), IL-21, IL-15, and L-arginine; and
  - d) administering the therapeutic population of gene-edited TILs to the patient.
352. The method of claim 351, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
353. The method of claim 351 or 352, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.

354. The method of claim 351 or 352, wherein the L-arginine is present at a concentration of about 5 mM.
355. The method of any one of claims 351-354, wherein the second cell culture medium comprises an NAD<sup>+</sup> booster.
356. The method of claim 355, wherein the NAD<sup>+</sup> booster is selected from the group consisting of L-Trp, NR, NMN, NAD<sup>+</sup>, NAM and P7C3 activator.
357. The method of claim 355, wherein the NAD<sup>+</sup> booster is NAD<sup>+</sup>.
358. The method of claim 357, wherein the NAD<sup>+</sup> is present at a concentration of about 50-75  $\mu$ M.
359. The method of any one of claims 351-358, wherein the first expansion is performed over a period of about 3-14 days.
360. The method of any one of claims 351-358, wherein the first expansion is performed over a period of about 11 days.
361. The method of any one of claims 351-360, wherein the second expansion is performed over a period of about 7-14 days.
362. The method of any one of claims 351-360, wherein the second expansion is performed over a period of about 11 days.
363. The method of any one of claims 351-362, wherein the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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.
364. The method of any one of claims 351-363, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient.
365. The method of claim 364, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.

366. The method of claim 364, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.
367. The method of claim 364, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day.
368. The method of any one of claims 365-367, wherein the cyclophosphamide is administered with mesna.
369. The method of any one of claims 351-368, further comprising a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient.
370. The method of any one of claims 351-368, further comprising a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient.
371. The method of claim 369 or 370, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.
372. The method of any one of claims 351-368, comprising no step of treating the patient with an IL-2 regimen.
373. A method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:
- (a) adding processed tumor fragments from a tumor resected from a patient into a closed system to obtain a first population of TILs;
  - (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11 days to obtain

the second population of TILs, and wherein the transition from step (a) to step (b) occurs without opening the system;

(c) performing a second expansion by culturing the second population of TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) harvesting the therapeutic population of TILs obtained from step (c), wherein the transition from step (c) to step (d) occurs without opening the system, and wherein the therapeutic population of TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

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

(f) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

374. A method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprising:

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

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

(c) performing a second expansion by culturing the second population of TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the

second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) harvesting the therapeutic population of TILs obtained from step (c), wherein the transition from step (c) to step (d) occurs without opening the system, and wherein the therapeutic population of TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

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

(f) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

375. A method for making a therapeutic population of gene-edited TILs comprising:

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

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

(c) transducing the second population of TILs with the recombinant lentiviral particle of any one of claims 42-46 to produce a population of gene-edited TILs, wherein the transition from step (b) to step (c) occurs without opening the system;

(d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, wherein the second expansion is performed in a closed container

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

(e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system, and wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

(f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, wherein the transfer from step (e) to (f) occurs without opening the system, and

(g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

376. A method for making a therapeutic population of gene-edited TILs comprising:

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

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

(c) transducing the second population of TILs with the recombinant lentiviral particle of any one of claims 42–46 to produce a population of gene-edited TILs, wherein the transition from step (b) to step (c) occurs without opening the system;

(d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;

(e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system, and wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

(f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, wherein the transfer from step (e) to (f) occurs without opening the system, and

(g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.

377. A method for making a therapeutic population of gene-edited TILs comprising:

(a) processing a tumor resected from a patient to obtain a first population of TILs;

(b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, and wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs;

(c) transducing the second population of TILs with the recombinant lentiviral particle of any one of claims 42-46 to produce a population of gene-edited TILs;

(d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, and wherein the second expansion is performed in a closed container providing a second gas-permeable surface area;

(e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;

(f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, and

- (g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.
378. A method for making a therapeutic population of gene-edited TILs comprising:
- (a) processing a tumor resected from a patient to obtain a first population of TILs;
  - (b) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, and wherein the first expansion is performed for about 3-11 days to obtain the second population of TILs;
  - (c) transducing the second population of TILs with the recombinant lentiviral particle of any one of claims 42-46 to produce a population of gene-edited TILs;
  - (d) performing a second expansion by culturing the population of gene-edited TILs in a second cell culture medium comprising IL-21, IL-15, L-arginine, NAD<sup>+</sup>, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11 days to obtain the third population of TILs, wherein the third population of TILs is the therapeutic population of gene-edited TILs, and wherein the second expansion is performed in a closed container providing a second gas-permeable surface area;
  - (e) harvesting the therapeutic population of gene-edited TILs obtained from step (d), wherein the therapeutic population of gene-edited TILs harvested comprises sufficient TILs for a therapeutically effective dosage of the TILs;
  - (f) transferring the harvested therapeutic population of gene-edited TILs from step (e) to an infusion bag, and
  - (g) cryopreserving the infusion bag comprising the harvested TIL population using a cryopreservation process.
379. The method of any one of claims 373-378, wherein the second cell culture medium comprises IL-15 at a concentration of about 10 ng/mL and IL-21 at a concentration of about 10 ng/mL.
380. The method of any one of claims 373-379, wherein the L-arginine is present at a concentration of about 1 mM to about 10 mM.
381. The method of any one of claims 373-379, wherein the L-arginine is present at a



concentration of about 5 mM.

382. The method of any one of claims 374, 376, 378-381, wherein the NAD<sup>+</sup> is present at a concentration of about 50-75  $\mu$ M.
383. The method of any one of claims 373-382, wherein the first expansion is performed over a period of about 3-11 days.
384. The method of any one of claims 373-382, wherein the first expansion is performed over a period of about 11 days.
385. The method of any one of claims 373-384, wherein the second expansion is performed over a period of about 7-11 days.
386. The method of any one of claims 373-384, wherein the second expansion is performed over a period of about 11 days.
387. The method of any one of claims 373-386, further comprising activating the population of TILs for 1 day or 2 days before transducing the population of TILs with the recombinant lentiviral particle.
388. The method of claim 387, wherein the activating step comprises contacting the population of TILs with TransAct.
389. The method of claim 387, wherein the activating step comprises contacting the population of TILs with TransAct at a ratio of 1:100.
390. The method of any one of claims 373-389, wherein the transducing step is conducted at a TIL concentration of  $10^5$  cells/mL.
391. The method of any one of claims 373-390, wherein the transducing step is conducted at a multiplicity of infection (MOI) of about 10 to about 40.
392. The method of any one of claims 373-391, wherein the transducing step is conducted in the presence of RetroNectin or Vectofusin-1.
393. The method of any one of claims 373-392, wherein the transducing step comprises centrifugation.
394. The method of any one of claims 373-393, wherein the transducing step is conducted in the presence of Lentibooster.
395. The method of any one of claims 373-394, further comprising resting the population

of TILs for 2 day or 3 days after the transducing step.

396. A therapeutic population of gene-edited TILs produced by the method of any one of claims 373-395.
397. A pharmaceutical composition comprising the therapeutic population of gene-edited TILs of claim 396.
398. Use of the therapeutic population of TILs of claim 349, or the therapeutic population of gene-edited TILs of any one of claims 172-184 and 396, for treating cancer in a patient in need thereof, comprising administering the therapeutic population of TILs or the therapeutic population of gene-edited TILs to the patient.
399. The use of claim 398, wherein the cancer is selected from the group consisting of melanoma (including mucosal melanoma, uveal melanoma, cutaneous melanoma, choroidal melanoma, ciliary body melanoma, or iris melanoma), ovarian cancer, cervical cancer, endometrial 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.
400. The use of claim 398 or 399, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs to the patient.
401. The use of claim 400, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.
402. The use of claim 400, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for three days.
403. The use of claim 400, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m<sup>2</sup>/day and fludarabine at a dose of 25 mg/m<sup>2</sup>/day for two days followed by administration of fludarabine at a dose of 25 mg/m<sup>2</sup>/day for one day.

404. The use of any one of claims 401-403, wherein the cyclophosphamide is administered with mesna.
405. The use of any one of claims 398-404, further comprising a step of treating the patient with an IL-2 regimen starting on the day after the administration of TILs to the patient.
406. The use of any one of claims 398-404, further comprising a step of treating the patient with an IL-2 regimen starting on the same day as administration of TILs to the patient.
407. The use of claim 405 or 406, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.
408. The method of any one of claims 398-404, comprising no step of treating the patient with an IL-2 regimen.