Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3231018 A1 2023/03/16

(21) 3 231 018

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) **Date de dépôt PCT/PCT Filing Date:** 2022/09/08
(87) **Date publication PCT/PCT Publication Date:** 2023/03/16
(85) **Entrée phase nationale/National Entry:** 2024/03/05
(86) **N° demande PCT/PCT Application No.:** US 2022/076136
(87) **N° publication PCT/PCT Publication No.:** 2023/039488
(30) **Priorités/Priorities:** 2021/09/09 (US63/242,373); 2021/12/09 (US63/287,670); 2022/03/21 (US63/322,190); 2022/06/22 (US63/354,605); 2022/08/01 (US63/394,248)

(51) **Cl.Int./Int.Cl.** ***A61K 35/17*** (2015.01), ***C12N 15/90*** (2006.01)

(71) **Demandeurs/Applicants:**
IOVANCE BIOTHERAPEUTICS, INC., US;
VEERAPATHRAN, ANAND, US;
WARDELL, SETH, US

(72) **Inventeurs/Inventors:**
VEERAPATHRAN, ANAND, US;
WARDELL, SETH, US

(74) **Agent:** BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) **Titre : PROCEDES DE PRODUCTION DE PRODUITS TIL PAR INACTIVATION DE PD-1 AVEC TALEN**
(54) **Title: PROCESSES FOR GENERATING TIL PRODUCTS USING PD-1 TALEN KNOCKDOWN**

(57) **Abrégé/Abstract:**
The present invention provides improved methods for expanding TILs and producing therapeutic populations of TILs, including methods for gene-editing at least a portion of the TILs to enhance their therapeutic efficacy. The methods lead to improved efficacy, improved phenotype, and increased metabolic health of the TILs in a shorter time period, while allowing for reduced microbial contamination as well as decreased costs. Such TILs find use in therapeutic treatment regimens.

Date Submitted: 2024/03/05

CA App. No.: 3231018

Abstract:

The present invention provides improved methods for expanding TILs and producing therapeutic populations of TILs, including methods for gene-editing at least a portion of the TILs to enhance their therapeutic efficacy. The methods lead to improved efficacy, improved phenotype, and increased metabolic health of the TILs in a shorter time period, while allowing for reduced microbial contamination as well as decreased costs. Such TILs find use in therapeutic treatment regimens.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 4
CONTENANT LES PAGES 1 À 224

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 4
CONTAINING PAGES 1 TO 224

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

PROCESSES FOR GENERATING TIL PRODUCTS USING PD-1 TALEN KNOCKDOWN

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 63/242,373, filed September 9, 2021, 63/287,670, filed December 9, 2021, 63/322,190, filed March 21, 2022, 63/354,605, filed June 22, 2022, and 63/394,248, filed August 1, 2022, the disclosures of which are herein incorporated in their entireties.

BACKGROUND OF THE INVENTION

[0002] Treatment of bulky, refractory cancers using adoptive autologous transfer of tumor infiltrating lymphocytes (TILs) represents a powerful approach to therapy for patients with poor prognoses. Gattinoni, *et al.*, *Nat. Rev. Immunol.* **2006,** *6,* 383-393. TILs are dominated by T cells, and IL-2-based TIL expansion followed by a "rapid expansion process" (REP) has become a preferred method for TIL expansion because of its speed and efficiency. Dudley, *et al.*, *Science* **2002,** *298,* 850-54; Dudley, *et al.*, *J. Clin. Oncol.* **2005,** *23,* 2346-57; Dudley, *et al.*, *J. Clin. Oncol.* **2008**, *26,* 5233-39; Riddell, *et al.*, *Science* **1992,** *257,* 238-41; Dudley, *et al.*, *J. Immunother.* **2003**, *26,* 332-42. A number of approaches to improve responses to TIL therapy in melanoma and to expand TIL therapy to other tumor types have been explored with limited success, and the field remains challenging. Goff, *et al., J. Clin. Oncol.* **2016,** *34,* 2389-97; Dudley, *et al., J. Clin. Oncol.* **2008**, *26,* 5233-39; Rosenberg, *et al., Clin. Cancer Res.* **2011,** *17,* 4550-57. Combination studies with single immune checkpoint inhibitors have also been described, but further studies are ongoing and additional methods of treatment are needed (Kverneland, et al., *Oncotarget*, **2020**, 11(22), 2092-2105).

[0003] Furthermore, current TIL manufacturing and treatment processes are limited by length, cost, sterility concerns, and other factors described herein such that the potential to treat patients which are refractory to checkpoint inhibitor therapies has been severely limited. There is an urgent need to provide TIL manufacturing processes and therapies based on such processes that are appropriate for use in treating patients for whom very few or no viable treatment options remain. The present invention meets this need by providing a shortened manufacturing process for use in generating TILs.

[0004] The present invention provides improved and/or shortened processes and methods for expanding TILs and producing therapeutic populations of TILs, including methods for gene-editing at least a portion of the therapeutic population of TILs to enhance their therapeutic effect.

BRIEF SUMMARY OF THE INVENTION

[0005] Provided herein are methods for expanding TILs and producing therapeutic populations of TILs, including methods for gene-editing at least a portion of the TILs to enhance their therapeutic efficacy.

[0006] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 agonist and anti-CD28 agonist beads or antibodies, for 1-7 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and

(e) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0007] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained and/or received from a tumor tissue resected from a subject or patient in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;

(b) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 agonist and anti-CD28 agonist beads or antibodies, for 1-7 days, to produce a third population of TILs;

(c) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and

(d) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0008] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 8 days;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 agonist and anti-CD28 agonist beads or antibodies, for 1-6 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(e) performing a rapid second expansion of the fourth population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[0009] In some embodiments, provided herein is a method of expanding tumor infiltrating lymphocytes into a therapeutic population of TILs, the method comprising the steps of:

(a) obtaining and/or receiving a first population of TILs from a sample of tumor tissue produced by surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining tumor tissue from a patient or subject;

(b) adding the tumor tissue into a closed system and performing a first expansion by culturing the first population of TILs in a first 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 3-9 days to obtain the second population of TILs;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or CD3 agonist and CD28 agonist beads or antibodies, for 1-7 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(e) performing a second expansion by culturing the fourth population of TILs in a second cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fifth population of TILs, wherein the second expansion is performed for about 5-15 days to obtain the fifth population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, wherein the fifth population of TILs is a therapeutic population of TILs; and

(f) harvesting the therapeutic population of TILs obtained from step (e), wherein each of steps (b) to (f) is performed in a closed, sterile system, and wherein the transition from step (b) to step (c), the transition from step (c) to step (d), the transition from step (d) to step (e) and/or the transition from step (e) to step (f) occurs without opening the system.

[0010] In some embodiments, the method further comprises:

[0011] digesting in an enzyme media the tumor tissue to produce a tumor digest.

[0012] In some embodiments, the enzymatic media comprises a DNase.

[0013] In some embodiments, the enzymatic media comprises a collagenase.

[0014] In some embodiments, the enzymatic media comprises a neutral protease.

[0015] In some embodiments, the enzymatic media comprises a hyaluronidase.

[0016] In some embodiments, the step of culturing or rapid second expansion of the fourth population of TILs is performed by culturing the fourth population of TILs in the second cell culture medium for a first period of about 1-7 days, at the end of the first period the fourth population of TILs is split into a plurality of subcultures, each of the subcultures is cultured in a third cell culture medium comprising IL-2 for a second period of about 3-7 days, and at the end of the second period the subcultures are combined to provide the expanded number of TILs or the therapeutic population of TILs.

[0017] In some embodiments, the first period of culturing is about 5 days.

[0018] In some embodiments, the second period of culturing is about 4 days.

[0019] In some embodiments, the second period of culturing is about 5 days.

[0020] In some embodiments, the step of activating the second population of TILs is performed using anti-CD3 agonist beads or antibodies.

[0021] In some embodiments, the step of activating the second population of TILs is performed using OKT-3.

[0022] In some embodiments, the step of activating the second population of TILs is performed using OKT-3 at 300 ng/mL.

[0023] In some embodiments, the step of activating the second population of TILs is performed using anti-CD3 agonist and anti-CD28 agonist beads or antibodies.

[0024] In some embodiments, the step of activating the second population of TILs is performed using TransAct.

[0025] In some embodiments, the step of activating the second population of TILs is performed using TransAct at 1:10, 1:17.5 or 1:100 dilution.

[0026] In some embodiments, the step of activating the second population of TILs is performed for about 2 days.

[0027] In some embodiments, the step of activating the second population of TILs is performed for about 3 days.

[0028] In some embodiments, the step of activating the second population of TILs is performed for about 4 days.

[0029] In some embodiments, the step of activating the second population of TILs is performed for about 5 days.

[0030] In some embodiments, the step of culturing the first population of TILs is performed for about 3 days.

[0031] In some embodiments, the step of culturing the first population of TILs is performed for about 5 days.

[0032] In some embodiments, the step of culturing the first population of TILs is performed for about 7 days.

[0033] In some embodiments, the step of culturing the fourth population of TILs is performed for about 8 days.

[0034] In some embodiments, the step of culturing the fourth population of TILs is performed for about 9 days.

[0035] In some embodiments, the step of culturing the fourth population of TILs is performed for about 8-9 days.

[0036] In some embodiments, the step of culturing the fourth population of TILs is performed for about 10 days.

[0037] In some embodiments, the step of culturing the fourth population of TILs is performed for about 8-10 days.

[0038] In some embodiments, all steps are completed within a period of about 22 days.

[0039] In some embodiments, all steps are completed within a period of about 19-22 days.

[0040] In some embodiments, all steps are completed within a period of about 19-20 days.

[0041] In some embodiments, all steps are completed within a period of about 20-22 days.

[0042] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(d) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0043] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained and/or received from a tumor tissue resected from a subject or patient in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(c) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0044] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 8 days;

(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(d) performing a rapid second expansion of the third population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[0045] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) performing an initial expansion (or priming first expansion) of a first population of TILs obtained and/or received from a tumor tissue resected from a subject or patient in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 8 days;

(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(c) performing a rapid second expansion of the third population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[0046] In some embodiments, the method further comprises:

[0047] digesting in an enzyme media the tumor tissue to produce a tumor digest.

[0048] In some embodiments, the enzymatic media comprises a DNase.

[0049] In some embodiments, the enzymatic media comprises a collagenase.

[0050] In some embodiments, the enzymatic media comprises a neutral protease.

[0051] In some embodiments, the enzymatic media comprises a hyaluronidase.

[0052] In some embodiments, provided herein is a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained by digesting in an enzyme media a tumor tissue resected from a subject or patient to produce a tumor digest in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(c) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0053] In some embodiments, the step of culturing or initial expansion of the first population of TILs comprises culturing the first population of TILs in the first cell culture medium comprising IL-2 for about 3 days followed by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3 for 2-6 days.

[0054] In some embodiments, the step of culturing or rapid second expansion of the third population of TILs is performed by culturing the third population of TILs in the second cell culture medium for a first period of about 1-7 days, at the end of the first period the third population of TILs is split into a plurality of subcultures, each of the subcultures is cultured in a third cell culture medium comprising IL-2 for a second period of about 3-7 days, and at the end of the second period the subcultures are combined to provide the expanded number of TILs.

[0055] In some embodiments, the first period of culturing is about 5 days.

[0056] In some embodiments, the second period of culturing is about 4 days.

[0057] In some embodiments, the second period of culturing is about 5 days.

[0058] In some embodiments, the step of culturing the first population of TILs is performed for about 3 days.

[0059] In some embodiments, the step of culturing the first population of TILs is performed for about 5 days.

[0060] In some embodiments, the step of culturing the first population of TILs is performed for about 7 days.

[0061] In some embodiments, the step of culturing the third population of TILs is performed for about 8 days.

[0062] In some embodiments, the step of culturing the third population of TILs is performed for about 9 days.

[0063] In some embodiments, the step of culturing the third population of TILs is performed for about 8-9 days.

[0064] In some embodiments, the step of culturing the third population of TILs is performed for about 10 days.

[0065] In some embodiments, the step of culturing the third population of TILs is performed for about 8-10 days.

[0066] In some embodiments, all steps are completed within a period of about 22 days.

[0067] In some embodiments, all steps are completed within a period of about 20 days.

[0068] In some embodiments, all steps are completed within a period of about 22 days.

[0069] In some embodiments, all steps are completed within a period of about 19-22 days.

[0070] In some embodiments, all steps are completed within a period of about 19-20 days.

[0071] In some embodiments, all steps are completed within a period of about 20-22 days.

[0072] In some embodiments, all steps are completed within a period of about 16-18 days.

[0073] In some embodiments, in the step of culturing or initial expansion of the first population of TILs in the first culture medium further comprises anti-CD3 and anti-CD28 beads or antibodies.

[0074] In some embodiments, the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct.

[0075] In some embodiments, the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct at 1:10, 1:17.5 or 1:100 dilution.

[0076] In some embodiments, the first culture medium comprises OKT-3 at 300 ng/mL.

[0077] In some embodiments, the step of culturing or initial expansion of the first population of TILs comprises culturing the first population of TILs in the first cell culture medium comprising IL-2 and anti-CD3 and anti-CD28 beads or antibodies for about 3 days followed by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3 for 2-4 days.

[0078] In some embodiments, the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct.

[0079] In some embodiments, the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct at 1:10, 1:17.5 or 1:100 dilution.

[0080] In some embodiments, the first culture medium comprises OKT-3 at 300 ng/mL.

[0081] In some embodiments, the expanded number of TILs comprises a therapeutic population of TILs.

[0082] In some embodiments, the step of gene-editing at least a portion of the second or third population of TILs comprises performing a sterile electroporation step on the second or third population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor.

[0083] In some embodiments, the step of gene-editing at least a portion of the second or third population of TILs comprises performing a sterile electroporation step on the second or third population of TILs, wherein the sterile electroporation step mediates the transfer of at least two gene editors.

[0084] In some embodiments, the electroporation step consists of a single electroporation event that mediates the transfer of the at least two gene editors.

[0085] In some embodiments, in the electroporation step for each of the at least two gene editors is transferred individually by an electroporation event independently of the transfer of any other gene editor.

[0086] In some embodiments, the electroporation step further comprises a rest period after each electroporation event.

[0087] In some embodiments, the electroporation step comprises a first electroporation event that mediates the transfer of a first gene editor for modulating expression of a first protein, a first rest period, a second electroporation event that mediates the transfer of a second gene editor for modulating expression of a second protein, and a second rest period, wherein the first and second rest periods are the same or different.

[0088] In some embodiments, the first and second rest periods comprise incubating the third or fourth population of TILs in the second cell culture medium comprising IL-2 and/or IL-15.

[0089] In some embodiments, the first and second rest periods comprise incubating the third or fourth population of TILs in the second cell culture medium comprising IL-2 at 300 IU/mL, 1000 IU/mL or 6000 IU/mL.

[0090] In some embodiments, the first and second rest periods comprise incubating the third or fourth population of TILs in the second cell culture medium comprising IL-15 at 15 ng/mL.

[0091] In some embodiments, the first and second rest periods comprise incubating the third or fourth population of TILs at about 30-40 °C with about 5% CO_2.

[0092] In some embodiments, the first and second rest periods comprise incubating the third or fourth population of TILs at about 25, 28, 30, 32, 35 or 37 °C with about 5% CO_2.

[0093] In some embodiments, the first and second rest periods are independently about 10 hours to 5 days.

[0094] In some embodiments, the first and second rest periods are independently about 10 hours to 3 days.

[0095] In some embodiments, the first rest period is about 1 to 3 days.

[0096] In some embodiments, the first rest period is about 3 days.

[0097] In some embodiments, the second rest period is about 10 hours to 1 day.

[0098] In some embodiments, the second rest period is about 12 hours to 24 hours.

[0099] In some embodiments, the second rest period is about 15 hours to about 18 hours.

[00100] In some embodiments, the second rest period comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about 15 hours to 23 hours at about 30°C.

[00101] In some embodiments, the second rest period comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about for about one hour at 37°C followed by about 15 hours to 23 hours at about 30°C.

[00102] In some embodiments, the second rest period comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 15 hours to 22 hours at about 30°C.

[00103] In some embodiments, the first rest period is about 3 days and the second rest period is about 10 to 16 hours.

[00104] In some embodiments, the electroporation step is preceded by washing the second or third population of TILs in a cytoporation buffer.

[00105] In some embodiments, the at least one gene editor is a TALE nuclease system for modulating the expression of at least one protein.

[00106] In some embodiments, the at least one gene editor comprises a TALE nuclease system that modulates expression of PD-1.

[00107] In some embodiments, the at least one gene editor comprises a TALE nuclease system that modulates expression of CTLA-4.

[00108] In some embodiments, the at least one gene editor comprises a TALE nuclease system that modulates expression of LAG-3.

[00109] In some embodiments, the at least one gene editor comprises a TALE nuclease system that modulates expression of CISH.

[00110] In some embodiments, the at least one gene editor comprises a TALE nuclease system that modulates expression of CBL-B.

[00111] In some embodiments, the at least one gene editor comprises a TALE nuclease system that modulates expression of TIGIT.

[00112] In some embodiments, the at least two gene editors comprise a first gene editor comprising a first TALE nuclease system for modulating expression of a first protein and a second gene editor comprising a second TALE nuclease system for modulating expression of a second protein.

[00113] In some embodiments, the first and second TALE nuclease systems modulate expression of PD-1, CTLA-4, LAG-3, CISH, TIGIT and/or CBL-B.

[00114] In some embodiments, the first and second TALE nuclease systems modulate expression of PD-1 and CTLA-4.

[00115] In some embodiments, the first and second TALE nuclease systems modulate expression of PD-1 and LAG-3.

[00116] In some embodiments, the first and second TALE nuclease systems modulate expression of PD-1 and CISH.

[00117] In some embodiments, the first and second TALE nuclease systems modulate expression of PD-1 and CBL-B.

[00118] In some embodiments, the first and second TALE nuclease systems modulate expression of PD-1 and TIGIT.

[00119] In some embodiments, the first and second TALE nuclease systems modulate expression of CTLA-4 and LAG-3.

[00120] In some embodiments, the first and second TALE nuclease systems modulate expression of CTLA-4 and CISH.

[00121] In some embodiments, the first and second TALE nuclease systems modulate expression of CTLA-4 and CBL-B.

[00122] In some embodiments, the first and second TALE nuclease systems modulate expression of LAG-3 and CISH.

[00123] In some embodiments, the first and second TALE nuclease systems modulate expression of LAG-3 and CBL-B.

[00124] In some embodiments, the first and second TALE nuclease systems modulate expression of CISH and CBL-B.

[00125] In some embodiments, the first protein and the second protein are independently selected from the group consisting of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, with the proviso that the first protein and the second protein are different.

[00126] In some embodiments, the first protein and the second protein are selected from the group consisting of PD-1 and CTLA-4.

[00127] In some embodiments, the first protein and the second protein are selected from the group consisting of PD-1 and LAG-3.

[00128] In some embodiments, the first protein and the second protein are selected from the group consisting of PD-1 and CISH.

[00129] In some embodiments, the first protein and the second protein are selected from the group consisting of PD-1 and CBL-B.

[00130] In some embodiments, the first protein and the second protein are selected from the group consisting of PD-1 and TIGIT.

[00131] In some embodiments, the first protein and the second protein are selected from the group consisting of CTLA-4 and LAG-3.

[00132] In some embodiments, the first protein and the second protein are selected from the group consisting of CTLA-4 and CISH.

[00133] In some embodiments, the first protein and the second protein are selected from the group consisting of CTLA-4 and CBL-B.

[00134] In some embodiments, the first protein and the second protein are selected from the group consisting of LAG-3 and CISH.

[00135] In some embodiments, the first protein and the second protein are selected from the group consisting of LAG-3 and CBL-B.

[00136] In some embodiments, the first protein and the second protein are selected from the group consisting of CISH and CBL-B.

[00137] In some embodiments, the first protein is PD-1 and the second protein is CTLA-4.

[00138] In some embodiments, the first protein is CTLA-4 and the second protein is PD-1.

[00139] In some embodiments, the first protein is PD-1 and the second protein is LAG-3.

[00140] In some embodiments, the first protein is LAG-3 and the second protein is PD-1.

[00141] In some embodiments, the first protein is PD-1 and the second protein is CISH.

[00142] In some embodiments, the first protein is CISH and the second protein is PD-1.

[00143] In some embodiments, the first protein is PD-1 and the second protein is CBL-B.

[00144] In some embodiments, the first protein is CBL-B and the second protein is PD-1.

[00145] In some embodiments, the first protein is PD-1 and the second protein is TIGIT.

[00146] In some embodiments, the first protein is TIGIT and the second protein is PD-1.

[00147] In some embodiments, the first protein is CTLA-4 and the second protein is LAG-3.

[00148] In some embodiments, the first protein is LAG-3 and the second protein is CTLA-4.

[00149] In some embodiments, the first protein is CTLA-4 and the second protein is CISH.

[00150] In some embodiments, the first protein is CISH and the second protein is CTLA-4.

[00151] In some embodiments, the first protein is CTLA-4 and the second protein is CBL-B.

[00152] In some embodiments, the first protein is CBL-B and the second protein is CTLA-4.

[00153] In some embodiments, the first protein is LAG-3 and the second protein is CISH.

[00154] In some embodiments, the first protein is CISH and the second protein is LAG-3.

[00155] In some embodiments, the first protein is LAG-3 and the second protein is CBL-B.

[00156] In some embodiments, the first protein is CBL-B and the second protein is LAG-3.

[00157] In some embodiments, the first protein is CISH and the second protein is CBL-B.

[00158] In some embodiments, the first protein is CBL-B and the second protein is CISH.

[00159] In some embodiments, the first protein or the second protein is PD-1.

[00160] In some embodiments, the first protein or the second protein is CTLA-4.

[00161] In some embodiments, the first protein or the second protein is LAG-3.

[00162] In some embodiments, the first protein or the second protein is CISH.

[00163] In some embodiments, the first protein or the second protein is CBL-B.

[00164] In some embodiments, the first protein or the second protein is TIGIT.

[00165] In some embodiments, the first gene editor downregulates expression of the first protein and the second gene editor downregulates expression of the second protein.

[00166] In some embodiments, the antigen presenting cells (APCs) are PBMCs.

[00167] In some embodiments, the PBMCs are irradiated and allogeneic.

[00168] In some embodiments, the antigen-presenting cells are artificial antigen-presenting cells.

[00169] In some embodiments, the IL-2 concentration is about 10,000 IU/mL to about 5,000 IU/mL.

[00170] In some embodiments, the first cell culture medium and/or the second cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist.

[00171] In some embodiments, the tumor tissue is processed into multiple tumor fragments.

[00172] In some embodiments, the tumor fragments are added into the closed system.

[00173] In some embodiments, 150 or fewer of the fragments, 100 or fewer of the fragments, or 50 or fewer of the fragments are added into the closed system.167. A gene-edited population of tumor infiltrating lymphocytes (TILs) comprising an expanded population of TILs wherein the expression of at least one protein is modulated by a gene editor transferred into at least a portion of the expanded population of TILs.

[00174] In some embodiments, the gene editor is a TALE nuclease system for modulating the expression of the at least one protein.

[00175] In some embodiments, the at least one protein is PD-1.

[00176] In some embodiments, the at least one protein is CTLA-4.

[00177] In some embodiments, the at least one protein is LAG-3.

[00178] In some embodiments, the at least one protein is CISH.

[00179] In some embodiments, the at least one protein is CBL-B.

[00180] In some embodiments, the at least one protein is TIGIT.

[00181] In some embodiments, the expression of at least two proteins is modulated by at least two gene editors transferred into at least a portion of the expanded population of TILs, wherein the at least two gene editors comprise a first gene editor comprising a first TALE nuclease system for modulating expression of a first protein and a second gene editor comprising a second TALE nuclease system for modulating expression of a second protein.

[00182] In some embodiments, the first and second proteins are independently selected from the group consisting of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, with the proviso that the first protein and the second protein are different.

[00183] In some embodiments, the first and second proteins are selected from the group consisting of PD-1 and CTLA-4.

[00184] In some embodiments, the first and second proteins are selected from the group consistingn of PD-1 and LAG-3.

[00185] In some embodiments, the first and second proteins are selected from the group consisting of PD-1 and CISH.

[00186] In some embodiments, the first and second proteins are selected from the group consisting of PD-1 and CBL-B.

[00187] In some embodiments, the first and second proteins are selected from the group consisting of PD-1 and TIGIT.

[00188] In some embodiments, the first and second proteins are selected from the group consisting of CTLA-4 and LAG-3.

[00189] In some embodiments, the first and second proteins are selected from the group consisting of CTLA-4 and CISH.

[00190] In some embodiments, the first and second proteins are selected from the group consisting of CTLA-4 and CBL-B.

[00191] In some embodiments, the first and second TALE proteins are selected from the group consisting of LAG-3 and CISH.

[00192] In some embodiments, the first and second proteins are selected from the group consisting of LAG-3 and CBL-B.

[00193] In some embodiments, the first and second proteins are selected from the group consisting of CISH and CBL-B.

[00194] In some embodiments, the gene-edited population of TILs disclosed herein is mannufactured by a method disclosed herein.

[00195] In some embodiments, provided herein is a pharmaceutical composition comprising the gene edited population of TILs disclosed herein and a pharmaceutically acceptable carrier.

[00196] In some embodiments, provided herein is a method for treating a subject with cancer, the method comprising administering a therapeutically effective dose of the gene edited population of TILs disclosed herein.

[00197] In some embodiments, the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), renal cancer, and renal cell carcinoma.

[00198] In some embodiments, provided herein is a method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a

tumor sample obtained from the patient into multiple tumor fragments;

(b) adding the tumor fragments into a closed system and performing a first expansion by culturing the first population of TILs in a first 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 3-8 days to obtain the second population of TILs;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 and anti-CD28 agonist beads or antibodies, for 1-6 days, to produce a third population of TILs;

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

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

(g) performing a second expansion by culturing the third population of TILs in a second cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 5-15 days to obtain the third population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, wherein the fourth population of TILs is a therapeutic population of TILs;

(h) harvesting the therapeutic population of TILs obtained from step (e) to provide a harvested TIL population, wherein one or more of steps (a) to (h) are performed in a closed, sterile system;

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

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

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

wherein the electroporation step comprises the delivery of a Transcription Activator-Like Effector Nuclease (TALEN) system for inhibiting the expression of PD-1, CTLA-4, LAG-3, CISH, TIGIT and/or CBL-B.

[00199] In some embodiments, the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of PD-1.

[00200] In some embodiments, the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of CTLA-4.

[00201] In some embodiments, the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of LAG-3.

[00202] In some embodiments, the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of CISH.

[00203] In some embodiments, the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of CBL-B.

[00204] In some embodiments, the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of TIGIT.

[00205] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and CTLA-4.

[00206] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and LAG-3.

[00207] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and CISH.

[00208] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and CBL-B.

[00209] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and TIGIT.

[00210] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and LAG-3.

[00211] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and CISH.

[00212] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and CBL-B.

[00213] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and TIGIT.

[00214] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of LAG-3 and CISH.

[00215] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of LAG-3 and CBL-B.

[00216] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of LAG-3 and TIGIT.

[00217] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CISH and CBL-B.

[00218] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CISH and TIGIT.

[00219] In some embodiments, the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CBL-B and TIGIT.

[00220] In some embodiments, the therapeutically effective dosage of TILs is from about 1×10^{9} to about 1×10^{11} TILs.

[00221] In some embodiments, prior to administering a therapeutically effective dosage of the harvested TIL population in step (k), a non-myeloablative lymphodepletion regimen has been administered to the patient.

[00222] In some embodiments, the method further comprises the step of treating the patient with a high-dose IL-2 regimen starting on the day after administration of the therapeutically effective dosage of the harvested TIL population to the patient in step (k).

[00223] In some embodiments, the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), renal cancer, and renal cell carcinoma.

[00224] In some embodiments, the cancer is melanoma.

[00225] In some embodiments, the cancer is metastatic melanoma.

[00226] In some embodiments, the cancer is NSCLC.

[00227] In some embodiments, the cancer is metastatic NSCLC.

[00228] In some embodiments, the gene-editing causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs.

BRIEF DESCRIPTION OF THE DRAWINGS

[00229] **Figure 1:** Exemplary Gen 2 (process 2A) chart providing an overview of Steps A through F.

[00230] **Figure 2A-2C:** Process flow chart of an embodiment of Gen 2 (process 2A) for TIL manufacturing.

[00231] **Figure 3:** Shows a diagram of an embodiment of a cryopreserved TIL exemplary manufacturing process (~22 days).

[00232] **Figure 4:** Shows a diagram of an embodiment of Gen 2 (process 2A), a 22-day process for TIL manufacturing.

[00233] **Figure 5:** Comparison table of Steps A through F from exemplary embodiments of process 1C and Gen 2 (process 2A) for TIL manufacturing.

[00234] **Figure 6:** Detailed comparison of an embodiment of process 1C and an embodiment of Gen 2 (process 2A) for TIL manufacturing.

[00235] **Figure 7:** Exemplary Gen 3 type TIL manufacturing process.

[00236] **Figure 8A-8P: A)** Shows a comparison between the 2A process (approximately 22-day process) and an embodiment of the Gen 3 process for TIL manufacturing (approximately 14-days to 16-days process). **B)** Exemplary Process Gen 3 chart providing an overview of Steps A through F (approximately 14-days to 16-days process). **C)** Chart providing three exemplary Gen 3 processes with an overview of Steps A through F (approximately 14-days to 16-days process) for each of the three process variations. **D)** Exemplary modified Gen 2-like process providing an overview of Steps A through F (approximately 22-days process). **E-P)** Schematics of exemplary embodiments of the KO TIL TALEN process.

[00237]

[00238] **Figure 9:** Provides an experimental flow chart for comparability between Gen 2 (process 2A) versus Gen 3 processes.

[00239] **Figure 10:** Shows a comparison between various Gen 2 (process 2A) and the Gen 3.1 process embodiment.

[00240] **Figure 11:** Table describing various features of embodiments of the Gen 2, Gen 2.1 and Gen 3.0 process.

[00241] **Figure 12:** Overview of the media conditions for an embodiment of the Gen 3 process, referred to as Gen 3.1.

[00242] **Figure 13:** Table describing various features of embodiments of the Gen 2, Gen 2.1 and Gen 3.0 process.

[00243] **Figure 14:** Table comparing various features of embodiments of the Gen 2 and Gen 3.0 processes.

[00244] **Figure 15:** Table providing media uses in the various embodiments of the described expansion processes.

[00245] **Figure 16:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

[00246] **Figure 17:** Schematic of an exemplary embodiment of a method for expanding T cells from hematopoietic malignancies using Gen 3 expansion platform.

[00247] **Figure 18:** Provides the structures I-A and I-B. The cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from *e.g.*, 4-1BBL or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second trivalent protein through IgG1-Fc (including CH3 and CH2 domains) is then used to link two of the trivalent proteins together through disulfide bonds (small elongated ovals), stabilizing the structure and providing an agonists capable of bringing together the intracellular signaling domains of the six receptors and signaling proteins to form a signaling complex. The TNFRSF binding domains denoted as cylinders may be scFv domains comprising, *e.g.*, a V_H and a V_L chain connected by a linker that may comprise hydrophilic residues and Gly and Ser sequences for flexibility, as well as Glu and Lys for solubility.

[00248] **Figure 19:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

[00249] **Figure 20:** Provides a process overview for an exemplary embodiment of the Gen 3.1 process (a 16 day process).

[00250] **Figure 21:** Schematic of an exemplary embodiment of the Gen 3.1 Test process (a 16-17 day process).

[00251] **Figure 22:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

[00252] **Figure 23:** Comparison table for exemplary Gen 2 and exemplary Gen 3 processes.

[00253] **Figure 24:** Schematic of an exemplary embodiment of the Gen 3 process (a 16-17 day process) preparation timeline.

[00254] **Figure 25:** Schematic of an exemplary embodiment of the Gen 3 process (a 14-16 day process).

[00255] **Figure 26A-26B:** Schematic of an exemplary embodiment of the Gen 3 process (a 16 day process).

[00256] **Figure 27:** Schematic of an exemplary embodiment of the Gen 3 process (a 16 day process).

[00257] **Figure 28:** Comparison of Gen 2, Gen 2.1 and an embodiment of the Gen 3 process (a 16 day process).

[00258] **Figure 29:** Comparison of Gen 2, Gen 2.1 and an embodiment of the Gen 3 process (a 16 day process).

[00259] **Figure 30:** Gen 3 embodiment components.

[00260] **Figure 31:** Gen 3 embodiment flow chart comparison (Gen 3.0, Gen 3.1 control, Gen 3.1 test).

[00261] **Figure 32:** Shown are the components of an exemplary embodiment of the Gen 3 process (a 16-17 day process).

[00262] **Figure 33:** Acceptance criteria table.

[00263] **Figure 34:** Experimental flow diagram of full-scale PD-1 KO TIL TALEN process.

[00264] **Figure 35:** Experimental flow diagram of full-scale PD-1 KO TIL TALEN process.

[00265] **Figure 36A-36D:** Schematics of exemplary embodiments of the KO TIL TALEN process.

[00266] **Figure 37:** Schematic of an exemplary embodiment of the process described in Example 12.

[00267] **Figure 38A-38B:** In vivo efficacy of PDCD-1 KO TIL. **A)** Efficiency of PDCD-1 KO assessed by flow cytometry. **B)** hIL-2 NOG mice (n=14 per treatment group) engrafted with melanoma tumor cells were adoptively transferred with PDCD-1 KO or mock TIL. Anti–PD-1 antibody treatment combined with mock TIL was included as a control for PD-1/PD-L1 blockade. Statistical significance is denoted by $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$.

[00268] **Figure 39A-39E:** Analysis of TIL product. **A)** Viable Cell Dose, **B)** Purity, **C)** Identity, **D)** Potency, and **E)** PDCD-1 KO Efficiency of TIL Product.

[00269] **Figure 40A-40B:** Analysis of TIL product. **A)** TIL Differentiation and **B)** TIL Memory.

[00270] **Figure 41A-41B:** Expression of Activation- and Inhibitory-Related Markers on PDCD-1 KO TIL.

[00271] **Figure 42A-42B:** IL-2–Independent Proliferation Assay of PDCD-1 KO TIL Products.

[00272] **Figure 43:** Summary of Karyotyping Results From PDCD-1 KO TIL Products.

[00273] **Figure 44A-44B:** cell viability (Figure 44A) and fold recovery (Figure 44B) of cells before electroporation.

[00274] **Figure 45A-45B:** fold recovery (Figure 45A) and cell viability (Figure 45B) of cells after electroporation.

[00275] **Figure 46A-46C:** knockout efficiency on CD3+ (Figure 46A), CD8+ (Figure 46B), and CD4+ (Figure 46C) cells.

[00276] **Figure 47A-47B:** fold recovery (Figure 47A) and cell viability (Figure 47B) of cells after electroporation.

[00277] **Figure 48A-48B:** fold recovery (Figure 48A) and cell viability (Figure 48B) of cells after electroporation when 6000 IU/mL IL-2 was used.

[00278] **Figure 49A-49B:** fold recovery (Figure 49A) and cell viability (Figure 49B) of cells after electroporation when various conditions were used.

[00279] **Figure 50A-50C:** knockout efficiency on CD3+ (Figure 50A), CD8+ (Figure 50B), and CD4+ (Figure 50C) cells.

[00280] **Figure 51:** cell viability before electroporation.

[00281] **Figure 52:** fold recovery of cells before electroporation.

[00282] **Figure 53A-53B:** fold recovery (Figure 53A) and cell viability (Figure 53B) of cells after electroporation.

[00283] **Figure 54A-54C:** knockout efficiency on CD3+ (Figure 54A), CD8+ (Figure 54B), and CD4+ (Figure 54C) cells.

[00284] **Figure 55A-55B:** cell number (Figure 55A) and viability (Figure 55B) after various wash steps.

[00285] **Figure 56A-56B:** cell number after various spin conditions using PBS wash (Figure 56A) or Cyto wash (Figure 56B).

[00286] **Figure 57A-57B:** cell viability after various spin conditions using PBS wash (Figure 57A) or Cyto wash (Figure 57B).

[00287] **Figure 58A-58B:** total spin comparison cell number (Figure 58A) and total spin comparison cell viability (Figure 58B) of cells after various spin conditions.

[00288] **Figure 59:** total spin comparison percent cell loss after various spin conditions.

[00289] **Figure 60A-60C:** percent loss and viability during electroporation, specifically, percent cell loss in the wash step (Figure 60A), percent cell loss after electroporation (Figure 60B), and cell viability after electroporation.

[00290] **Figure 61A-61C:** knockout efficiency on CD3+ (Figure 61A), CD8+ (Figure 61B), and CD4+ (Figure 61C) cells.

[00291] **Figure 62A-62B:** cell viability (Figure 62A) and fold expansion (Figure 62B) of REP harvest.

[00292] **Figure 63A-63B:** percent cell loss (Figure 63A) and cell viability (Figure 63B) after electroporation.

[00293] **Figure 64A-64C:** knockout efficiency in CD3+ (Figure 63A), CD4+ (Figure 63B), and CD8+ (Figure 63C) cells.

[00294] **Figure 65A-65B:** fold expansion (Figure 65A) and cell viability (Figure 65B) of REP harvest.

[00295] **Figure 66A-66C:** cell growth (Figure 66A), first electroporation knockout efficiency (Figure 66B), and second electroporation knockout efficiency (Figure 66C).

[00296] **Figure 67:** percent growth over 3 day rest.

[00297] **Figure 68A-68C:** PD-1 Knockout Efficiency.

[00298] **Figure 69:** *PDCD1* gene modification by NGS.

[00299] **Figure 70A-70B:** distribution of TCR Vβ subtypes in bulk PD-1 KO TIL product and NE TIL in the CD3+PD-1- subset.

[00300] **Figure 71A-71B:** PD-1 KO TIL effector function as measured by MLR (Figure 71A) and polyfunctionality (Figure 71B).

[00301] **Figure 72:** in vivo anti-tumor activity of M1152 PD-1 KO TIL product.

[00302] **Figure 73A-73B:** TALEN protein persistence in autologous TIL as a function of time measured by western blot.

[00303] **Figure 74A-F:** Exemplary TIL manufacturing process.

[00304] **Figure 75A-B:** Schemas of the Phase 1/2 study described in Example 22.

[00305] **Figure 76:** summary of data described in Example 23.

[00306] **Figure 77A-D:** results from Demo Day Experiment of Example 23.

[00307] **Figure 78A-C:** Results from Neon Exp 1 of Example 23.

[00308] **Figure 79A-C:** Results from Xenon Exp 1 of Example 23.

[00309] **Figure 80A-B:** Results from Xenon Exp 3 of Example 23.

[00310] **Figure 81A-C:** Results from Xenon Exp 4 of Example 23.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

[0001] SEQ ID NO:1 is the amino acid sequence of the heavy chain of muromonab.

[0002] SEQ ID NO:2 is the amino acid sequence of the light chain of muromonab.

[0003] SEQ ID NO:3 is the amino acid sequence of a recombinant human IL-2 protein.

[0004] SEQ ID NO:4 is the amino acid sequence of aldesleukin.

[0005] SEQ ID NO:5 is an IL-2 form.

[0001] SEQ ID NO:6 is the amino acid sequence of nemvaleukin alfa.

[0002] SEQ ID NO:7 is an IL-2 form.

[0003] SEQ ID NO:8 is a mucin domain polypeptide.

[0004] SEQ ID NO:9 is the amino acid sequence of a recombinant human IL-4 protein.

[0005] SEQ ID NO:10 is the amino acid sequence of a recombinant human IL-7 protein.

[0006] SEQ ID NO:11 is the amino acid sequence of a recombinant human IL-15 protein.

[0007] SEQ ID NO:12 is the amino acid sequence of a recombinant human IL-21 protein.

[0008] SEQ ID NO:13 is an IL-2 sequence.

[0009] SEQ ID NO:14 is an IL-2 mutein sequence.

[0010] SEQ ID NO:15 is an IL-2 mutein sequence.

[0011] SEQ ID NO:16 is the HCDR1_IL-2 for IgG.IL2R67A.H1.

[0012] SEQ ID NO:17 is the HCDR2 for IgG.IL2R67A.H1.

[0013] SEQ ID NO:18 is the HCDR3 for IgG.IL2R67A.H1.

[0014] SEQ ID NO:19 is the HCDR1_IL-2 kabat for IgG.IL2R67A.H1.

[0015] SEQ ID NO:20 is the HCDR2 kabat for IgG.IL2R67A.H1.

[0016] SEQ ID NO:21 is the HCDR3 kabat for IgG.IL2R67A.H1.

[0017] SEQ ID NO:22 is the HCDR1_IL-2 clothia for IgG.IL2R67A.H1.

[0018] SEQ ID NO:23 is the HCDR2 clothia for IgG.IL2R67A.H1.

[0019] SEQ ID NO:24 is the HCDR3 clothia for IgG.IL2R67A.H1.

[0020] SEQ ID NO:25 is the HCDR1_IL-2 IMGT for IgG.IL2R67A.H1.

[0021] SEQ ID NO:26 is the HCDR2 IMGT for IgG.IL2R67A.H1.

[0022] SEQ ID NO:27 is the HCDR3 IMGT for IgG.IL2R67A.H1.

[0023] SEQ ID NO:28 is the V_H chain for IgG.IL2R67A.H1.

[0024] SEQ ID NO:29 is the heavy chain for IgG.IL2R67A.H1.

[0025] SEQ ID NO:30 is the LCDR1 kabat for IgG.IL2R67A.H1.

[0026] SEQ ID NO:31 is the LCDR2 kabat for IgG.IL2R67A.H1.

[0027] SEQ ID NO:32 is the LCDR3 kabat for IgG.IL2R67A.H1.

[0028] SEQ ID NO:33 is the LCDR1 chothia for IgG.IL2R67A.H1.

[0029] SEQ ID NO:34 is the LCDR2 chothia for IgG.IL2R67A.H1.

[0030] SEQ ID NO:35 is the LCDR3 chothia for IgG.IL2R67A.H1.

[0031] SEQ ID NO:36 is a V_L chain.

[0032] SEQ ID NO:37 is a light chain.

[0033] SEQ ID NO:38 is a light chain.

[0034] SEQ ID NO:39 is a light chain.

[0035] SEQ ID NO:40 is the amino acid sequence of human 4-1BB.

[0036] SEQ ID NO:41 is the amino acid sequence of murine 4-1BB.

[0037] SEQ ID NO:42 is the heavy chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0038] SEQ ID NO:43 is the light chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0039] SEQ ID NO:44 is the heavy chain variable region (V_H) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0040] SEQ ID NO:45 is the light chain variable region (V_L) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0041] SEQ ID NO:46 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0042] SEQ ID NO:47 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0043] SEQ ID NO:48 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0044] SEQ ID NO:49 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0045] SEQ ID NO:50 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0046] SEQ ID NO:51 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[0047] SEQ ID NO:52 is the heavy chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0048] SEQ ID NO:53 is the light chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0049] SEQ ID NO:54 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0050] SEQ ID NO:55 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0051] SEQ ID NO:56 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0052] SEQ ID NO:57 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0053] SEQ ID NO:58 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0054] SEQ ID NO:59 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0055] SEQ ID NO:60 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0056] SEQ ID NO:61 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[0057] SEQ ID NO:62 is an Fc domain for a TNFRSF agonist fusion protein.

[0058] SEQ ID NO:63 is a linker for a TNFRSF agonist fusion protein.

[0059] SEQ ID NO:64 is a linker for a TNFRSF agonist fusion protein.

[0060] SEQ ID NO:65 is a linker for a TNFRSF agonist fusion protein.

[0061] SEQ ID NO:66 is a linker for a TNFRSF agonist fusion protein.

[0062] SEQ ID NO:67 is a linker for a TNFRSF agonist fusion protein.

[0063] SEQ ID NO:68 is a linker for a TNFRSF agonist fusion protein.

[0064] SEQ ID NO:69 is a linker for a TNFRSF agonist fusion protein.

[0065] SEQ ID NO:70 is a linker for a TNFRSF agonist fusion protein.

[0066] SEQ ID NO:71 is a linker for a TNFRSF agonist fusion protein.

[0067] SEQ ID NO:72 is a linker for a TNFRSF agonist fusion protein.

[0068] SEQ ID NO:73 is an Fc domain for a TNFRSF agonist fusion protein.

[0069] SEQ ID NO:74 is a linker for a TNFRSF agonist fusion protein.

[0070] SEQ ID NO:75 is a linker for a TNFRSF agonist fusion protein.

[0071] SEQ ID NO:76 is a linker for a TNFRSF agonist fusion protein.

[0072] SEQ ID NO:77 is a 4-1BB ligand (4-1BBL) amino acid sequence.

[0073] SEQ ID NO:78 is a soluble portion of 4-1BBL polypeptide.

[0074] SEQ ID NO:79 is a heavy chain variable region (V_H) for the 4-1BB agonist antibody 4B4-1-1 version 1.

[0075] SEQ ID NO:80 is a light chain variable region (V_L) for the 4-1BB agonist antibody 4B4-1-1 version 1.

[0076] SEQ ID NO:81 is a heavy chain variable region (V_H) for the 4-1BB agonist antibody 4B4-1-1 version 2.

[0077] SEQ ID NO:82 is a light chain variable region (V_L) for the 4-1BB agonist antibody 4B4-1-1 version 2.

[0078] SEQ ID NO:83 is a heavy chain variable region (V_H) for the 4-1BB agonist antibody H39E3-2.

[0079] SEQ ID NO:84 is a light chain variable region (V_L) for the 4-1BB agonist antibody H39E3-2.

[0080] SEQ ID NO:85 is the amino acid sequence of human OX40.

[0081] SEQ ID NO:86 is the amino acid sequence of murine OX40.

[0082] SEQ ID NO:87 is the heavy chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0083] SEQ ID NO:88 is the light chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0084] SEQ ID NO:89 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0085] SEQ ID NO:90 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0086] SEQ ID NO:91 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0087] SEQ ID NO:92 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0088] SEQ ID NO:93 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0089] SEQ ID NO:94 is the light chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0090] SEQ ID NO:95 is the light chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0091] SEQ ID NO:96 is the light chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[0092] SEQ ID NO:97 is the heavy chain for the OX40 agonist monoclonal antibody 11D4.

[0093] SEQ ID NO:98 is the light chain for the OX40 agonist monoclonal antibody 11D4.

[0094] SEQ ID NO:99 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 11D4.

[0095] SEQ ID NO:100 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 11D4.

[0096] SEQ ID NO:101 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

[0097] SEQ ID NO:102 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

[0098] SEQ ID NO:103 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

[0099] SEQ ID NO:104 is the light chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

[00100] SEQ ID NO:105 is the light chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

[00101] SEQ ID NO:106 is the light chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

[00102] SEQ ID NO:107 is the heavy chain for the OX40 agonist monoclonal antibody 18D8.

[00103] SEQ ID NO:108 is the light chain for the OX40 agonist monoclonal antibody 18D8.

[00104] SEQ ID NO:109 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 18D8.

[00105] SEQ ID NO:110 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 18D8.

[00106] SEQ ID NO:111 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody 18D8.

[00107] SEQ ID NO:112 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

[00108] SEQ ID NO:113 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

[00109] SEQ ID NO:114 is the light chain CDR1 for the OX40 agonist monoclonal antibody 18D8.

[00110] SEQ ID NO:115 is the light chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

[00111] SEQ ID NO:116 is the light chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

[00112] SEQ ID NO:117 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody Hu119-122.

[00113] SEQ ID NO:118 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody Hu119-122.

[00114] SEQ ID NO:119 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.

[00115] SEQ ID NO:120 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

[00116] SEQ ID NO:121 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

[00117] SEQ ID NO:122 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.

[00118] SEQ ID NO:123 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

[00119] SEQ ID NO:124 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

[00120] SEQ ID NO:125 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody Hu106-222.

[00121] SEQ ID NO:126 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody Hu106-222.

[00122] SEQ ID NO:127 is the heavy chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.

[00123] SEQ ID NO:128 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

[00124] SEQ ID NO:129 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

[00125] SEQ ID NO:130 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.

[00126] SEQ ID NO:131 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

[00127] SEQ ID NO:132 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

[00128] SEQ ID NO:133 is an OX40 ligand (OX40L) amino acid sequence.

[00129] SEQ ID NO:134 is a soluble portion of OX40L polypeptide.

[00130] SEQ ID NO:135 is an alternative soluble portion of OX40L polypeptide.

[00131] SEQ ID NO:136 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 008.

[00132] SEQ ID NO:137 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 008.

[00133] SEQ ID NO:138 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 011.

[00134] SEQ ID NO:139 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 011.

[00135] SEQ ID NO:140 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 021.

[00136] SEQ ID NO:141 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 021.

[00137] SEQ ID NO:142 is the heavy chain variable region (V_H) for the OX40 agonist monoclonal antibody 023.

[00138] SEQ ID NO:143 is the light chain variable region (V_L) for the OX40 agonist monoclonal antibody 023.

[00139] SEQ ID NO:144 is the heavy chain variable region (V_H) for an OX40 agonist monoclonal antibody.

[00140] SEQ ID NO:145 is the light chain variable region (V_L) for an OX40 agonist monoclonal antibody.

[00141] SEQ ID NO:146 is the heavy chain variable region (V_H) for an OX40 agonist monoclonal antibody.

[00142] SEQ ID NO:147 is the light chain variable region (V_L) for an OX40 agonist monoclonal antibody.

[00143] SEQ ID NO:148 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00144] SEQ ID NO:149 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00145] SEQ ID NO:150 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00146] SEQ ID NO:151 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00147] SEQ ID NO:152 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[00148] SEQ ID NO:153 is the heavy chain variable region (V_H) for a humanized OX40 agonist monoclonal antibody.

[0006] SEQ ID NO:154 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00149] SEQ ID NO:155 is the light chain variable region (V_L) for a humanized OX40 agonist monoclonal antibody.

[00150] SEQ ID NO:156 is the heavy chain variable region (V_H) for an OX40 agonist monoclonal antibody.

[00151] SEQ ID NO:157 is the light chain variable region (V_L) for an OX40 agonist monoclonal antibody.

[00152] SEQ ID NO:158 is the heavy chain amino acid sequence of the PD-1 inhibitor nivolumab.

[00153] SEQ ID NO:159 is the light chain amino acid sequence of the PD-1 inhibitor nivolumab.

[00154] SEQ ID NO:160 is the heavy chain variable region (V_H) amino acid sequence of the PD-1 inhibitor nivolumab.

[00155] SEQ ID NO:161 is the light chain variable region (V_L) amino acid sequence of the PD-1 inhibitor nivolumab.

[00156] SEQ ID NO:162 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

[00157] SEQ ID NO:163 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

[00158] SEQ ID NO:164 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

[00159] SEQ ID NO:165 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

[00160] SEQ ID NO:166 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

[00161] SEQ ID NO:167 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

[00162] SEQ ID NO:168 is the heavy chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00163] SEQ ID NO:169 is the light chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00164] SEQ ID NO:170 is the heavy chain variable region (V_H) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00165] SEQ ID NO:171 is the light chain variable region (V_L) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00166] SEQ ID NO:172 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00167] SEQ ID NO:173 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00168] SEQ ID NO:174 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00169] SEQ ID NO:175 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00170] SEQ ID NO:176 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00171] SEQ ID NO:177 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00172] SEQ ID NO:178 is the heavy chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00173] SEQ ID NO:179 is the light chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00174] SEQ ID NO:180 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00175] SEQ ID NO:181 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00176] SEQ ID NO:182 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00177] SEQ ID NO:183 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00178] SEQ ID NO:184 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00179] SEQ ID NO:185 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00180] SEQ ID NO:186 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00181] SEQ ID NO:187 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00182] SEQ ID NO:188 is the heavy chain amino acid sequence of the PD-L1 inhibitor avelumab.

[00183] SEQ ID NO:189 is the light chain amino acid sequence of the PD-L1 inhibitor avelumab.

[00184] SEQ ID NO:190 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor avelumab.

[00185] SEQ ID NO:191 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor avelumab.

[00186] SEQ ID NO:192 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

[00187] SEQ ID NO:193 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

[00188] SEQ ID NO:194 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

[00189] SEQ ID NO:195 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

[00190] SEQ ID NO:196 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

[00191] SEQ ID NO:197 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

[00192] SEQ ID NO:198 is the heavy chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00193] SEQ ID NO:199 is the light chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00194] SEQ ID NO:200 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00195] SEQ ID NO:201 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00196] SEQ ID NO:202 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00197] SEQ ID NO:203 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00198] SEQ ID NO:204 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00199] SEQ ID NO:205 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00200] SEQ ID NO:206 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00201] SEQ ID NO:207 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00202] SEQ ID NO:208 is the heavy chain amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00203] SEQ ID NO:209 is the light chain amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00204] SEQ ID NO:210 is the heavy chain variable region (V_H) amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00205] SEQ ID NO:211 is the light chain variable region (V_L) amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00206] SEQ ID NO:212 is the heavy chain CDR1 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00207] SEQ ID NO:213 is the heavy chain CDR2 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00208] SEQ ID NO:214 is the heavy chain CDR3 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00209] SEQ ID NO:215 is the light chain CDR1 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00210] SEQ ID NO:216 is the light chain CDR2 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00211] SEQ ID NO:217 is the light chain CDR3 amino acid sequence of the CTLA-4 inhibitor ipilimumab.

[00212] SEQ ID NO:218 is the heavy chain amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00213] SEQ ID NO:219 is the light chain amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00214] SEQ ID NO:220 is the heavy chain variable region (V_H) amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00215] SEQ ID NO:221 is the light chain variable region (V_L) amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00216] SEQ ID NO:222 is the heavy chain CDR1 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00217] SEQ ID NO:223 is the heavy chain CDR2 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00218] SEQ ID NO:224 is the heavy chain CDR3 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00219] SEQ ID NO:225 is the light chain CDR1 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00220] SEQ ID NO:226 is the light chain CDR2 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00221] SEQ ID NO:227 is the light chain CDR3 amino acid sequence of the CTLA-4 inhibitor tremelimumab.

[00222] SEQ ID NO:228 is the heavy chain amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00223] SEQ ID NO:229 is the light chain amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00224] SEQ ID NO:230 is the heavy chain variable region (V_H) amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00225] SEQ ID NO:231 is the light chain variable region (V_L) amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00226] SEQ ID NO:232 is the heavy chain CDR1 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00227] SEQ ID NO:233 is the heavy chain CDR2 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00228] SEQ ID NO:234 is the heavy chain CDR3 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00229] SEQ ID NO:235 is the light chain CDR1 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00230] SEQ ID NO:236 is the light chain CDR2 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00231] SEQ ID NO:237 is the light chain CDR3 amino acid sequence of the CTLA-4 inhibitor zalifrelimab.

[00232] SEQ ID NO:238 is an exemplary Clo05 I nuclease domain amino acid sequence.

[00233] SEQ ID NO:239 is an exemplary piggyBac (PB) transposase enzyme amino acid sequence.

[00234] SEQ ID NO:240 is an exemplary Sleeping Beauty transposase enzyme amino acid sequence.

[00235] SEQ ID NO:241 is an exemplary hyperactive Sleeping Beauty (SB100X) transposase amino acid sequence.

I. Definitions

[0007] 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.

[0008] 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.

[0009] The term "*in vivo*" refers to an event that takes place in a subject's body.

[0010] 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.

[0011] 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.

[0012] 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.

[0013] By "tumor infiltrating lymphocytes" or "TILs" herein is meant a population of cells originally obtained as white blood cells that have left the bloodstream of a subject and migrated into a tumor. TILs include, but are not limited to, $CD8^+$ cytotoxic T cells (lymphocytes), Th1 and Th17 $CD4^+$ T cells, natural killer cells, dendritic cells and M1 macrophages. TILs include both primary and secondary TILs. "Primary TILs" are those that are obtained from patient tissue samples as outlined herein (sometimes referred to

as "freshly harvested"), and "secondary TILs" are any TIL cell populations that have been expanded or proliferated as discussed herein, including, but not limited to bulk TILs and expanded TILs ("REP TILs" or "post-REP TILs"). TIL cell populations can include genetically modified TILs.

[0014] By "population of cells" (including TILs) herein is meant a number of cells that share common traits. In general, populations generally range from 1 X 10^6 to 1 X 10^{10} in number, with different TIL populations comprising different numbers. For example, initial growth of primary TILs in the presence of IL-2 results in a population of bulk TILs of roughly 1×10^8 cells. REP expansion is generally done to provide populations of 1.5×10^9 to 1.5×10^{10} cells for infusion.

[0015] By "cryopreserved TILs" herein is meant that TILs, either primary, bulk, or expanded (REP TILs), are treated and stored in the range of about -150°C to -60°C. General methods for cryopreservation are also described elsewhere herein, including in the Examples. For clarity, "cryopreserved TILs" are distinguishable from frozen tissue samples which may be used as a source of primary TILs.

[0016] 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.

[0017] TILs can generally be defined either biochemically, using cell surface markers, or functionally, by their ability to infiltrate tumors and effect treatment. TILs can be generally categorized by expressing one or more of the following biomarkers: CD4, CD8, TCR αβ, CD27, CD28, CD56, CCR7, CD45Ra, CD95, PD-1, and CD25. Additionally and alternatively, TILs can be functionally defined by their ability to infiltrate solid tumors upon reintroduction into a patient.

[0018] The term "cryopreservation media" or "cryopreservation medium" refers to any medium that can be used for cryopreservation of cells. Such media can include media comprising 7% to 10% DMSO. Exemplary media include CryoStor CS10, Hyperthermasol, as well as combinations thereof. The term "CS10" refers to a cryopreservation medium which is obtained from Stemcell Technologies or from Biolife Solutions. The CS10 medium may be referred to by the trade name "CryoStor® CS10". The CS10 medium is a serum-free, animal component-free medium which comprises DMSO. In some embodiments, the CS10 medium comprises 10% DMSO.

[0019] The term "central memory T cell" refers to a subset of T cells that in the human are CD45R0+ and constitutively express CCR7 ($CCR7^{hi}$) and CD62L ($CD62^{hi}$). The surface phenotype of central memory T cells also includes TCR, CD3, CD127 (IL-7R), and IL-15R. Transcription factors for central memory T cells

include BCL-6, BCL-6B, MBD2, and BMI1. Central memory T cells primarily secret IL-2 and CD40L as effector molecules after TCR triggering. Central memory T cells are predominant in the CD4 compartment in blood, and in the human are proportionally enriched in lymph nodes and tonsils.

[0020] The term "effector memory T cell" refers to a subset of human or mammalian T cells that, like central memory T cells, are CD45R0+, but have lost the constitutive expression of CCR7 ($CCR7^{lo}$) and are heterogeneous or low for CD62L expression ($CD62L^{lo}$). The surface phenotype of central memory T cells also includes TCR, CD3, CD127 (IL-7R), and IL-15R. Transcription factors for central memory T cells include BLIMP1. Effector memory T cells rapidly secret high levels of inflammatory cytokines following antigenic stimulation, including interferon-γ, IL-4, and IL-5. Effector memory T cells are predominant in the CD8 compartment in blood, and in the human are proportionally enriched in the lung, liver, and gut. CD8+ effector memory T cells carry large amounts of perforin.

[0021] 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.

[0022] 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.

[0023] 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.

[0024] The terms "peripheral blood lymphocytes" and "PBLs" refer to T cells expanded from peripheral blood. In some embodiments, PBLs are separated from whole blood or apheresis product from a donor. In some embodiments, PBLs are separated from whole blood or apheresis product from a donor by positive or negative selection of a T cell phenotype, such as the T cell phenotype of CD3+ CD45+.

[0025] 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.

[0026] The term "OKT-3" (also referred to herein as "OKT3") refers to a monoclonal antibody or biosimilar or variant thereof, including human, humanized, chimeric, or murine antibodies, directed against the CD3 receptor in the T cell antigen receptor of mature T cells, and includes commercially-available forms such as OKT-3 (30 ng/mL, MACS GMP CD3 pure, Miltenyi Biotech, Inc., San Diego, CA, USA) and muromonab or variants, conservative amino acid substitutions, glycoforms, or biosimilars thereof. The amino acid sequences of the heavy and light chains of muromonab are given in Table 1 (SEQ ID NO:1 and SEQ ID NO:2). A hybridoma capable of producing OKT-3 is deposited with the American Type Culture Collection and assigned the ATCC accession number CRL 8001. A hybridoma capable of producing OKT-3 is also deposited with European Collection of Authenticated Cell Cultures (ECACC) and assigned Catalogue No. 86022706.

TABLE 1. Amino acid sequences of muromonab (exemplary OKT-3 antibody).

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

[0027] 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^6 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.

[00236] 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, and 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, phosphonotyrosine, tri-O-acetyl-GlcNAcp-serine, L-phosphoserine, phosphonoserine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, 2-amino-3-(phenylselanyl)propanoic, or selenocysteine. In some embodiments, the IL-2 conjugate has a decreased affinity to IL-2 receptor α (IL-2Rα) 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-2Rα 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-2Rα. In some

embodiments, the conjugating moiety comprises a water-soluble polymer. In some embodiments, the additional conjugating moiety comprises a water-soluble polymer. In some embodiments, each of the water-soluble polymers independently comprises polyethylene glycol (PEG), poly(propylene glycol) (PPG), copolymers of ethylene glycol and propylene glycol, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α-hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazolines (POZ), poly(N-acryloylmorpholine), or a combination thereof. In some embodiments, each of the water-soluble polymers independently comprises PEG. In some embodiments, the PEG is a linear PEG or a branched PEG. In some embodiments, each of the water-soluble polymers independently comprises a polysaccharide. In some embodiments, the polysaccharide comprises dextran, polysialic acid (PSA), hyaluronic acid (HA), amylose, heparin, heparan sulfate (HS), dextrin, or hydroxyethyl-starch (HES). In some embodiments, each of the water-soluble polymers independently comprises a glycan. In some embodiments, each of the water-soluble polymers independently comprises polyamine. In some embodiments, the conjugating moiety comprises a protein. In some embodiments, the additional conjugating moiety comprises a protein. In some embodiments, each of the proteins independently comprises an albumin, a transferrin, or a transthyretin. In some embodiments, each of the proteins independently comprises an Fc portion. In some embodiments, each of the proteins independently comprises an Fc portion of IgG. In some embodiments, the conjugating moiety comprises a polypeptide. In some embodiments, the additional conjugating moiety comprises a polypeptide. In some embodiments, each of the polypeptides independently comprises a XTEN peptide, a glycine-rich homoamino acid polymer (HAP), a PAS polypeptide, an elastin-like polypeptide (ELP), a CTP peptide, or a gelatin-like protein (GLK) polymer. In some embodiments, the isolated and purified IL-2 polypeptide is modified by glutamylation. In some embodiments, the conjugating moiety is directly bound to the isolated and purified IL-2 polypeptide. In some embodiments, the conjugating moiety is indirectly bound to the isolated and purified IL-2 polypeptide through a linker. In some embodiments, the linker comprises a homobifunctional linker. In some embodiments, the homobifunctional linker comprises Lomant's reagent dithiobis (succinimidylpropionate) DSP, 3′ 3′ -dithiobis(sulfosuccinimidyl proprionate) (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), bismaleimidohexane (BMH), aryl halide-containing compound (DFDNB), such as *e.g.* 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene, 4,4′-difluoro-3,3′-dinitrophenylsulfone (DFDNPS), bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED), formaldehyde, glutaraldehyde, 1,4-butanediol diglycidyl ether, adipic acid dihydrazide, carbohydrazide, o-toluidine, 3,3′-dimethylbenzidine, benzidine, α,α′-p-diaminodiphenyl, diiodo-p-xylene sulfonic acid, N,N′-ethylene-bis(iodoacetamide), or N,N′-hexamethylene-bis(iodoacetamide). In some embodiments, the linker comprises a heterobifunctional linker. In some embodiments, the heterobifunctional linker comprises N-succinimidyl 3-(2-pyridyldithio)propionate (sPDP), long-chain N-succinimidyl 3-(2-pyridyldithio)propionate (LC-sPDP), water-soluble-long-chain N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-sPDP), succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (sMPT), sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-sMPT), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBs), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBs), N-succinimidyl(4-iodoacteyl)aminobenzoate (sIAB), sulfosuccinimidyl(4-iodoacteyl)aminobenzoate (sulfo-sIAB), succinimidyl-4-(p-maleimidophenyl)butyrate (sMPB), sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (sulfo-sMPB), N-(γ-maleimidobutyryloxy)succinimide ester (GMBs), N-(γ-maleimidobutyryloxy) sulfosuccinimide ester (sulfo-GMBs), succinimidyl 6-((iodoacetyl)amino)hexanoate (sIAX), succinimidyl 6-[6-(((iodoacetyl)amino)hexanoyl)amino]hexanoate (sIAXX), succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (sIAC), succinimidyl 6-(((((4-iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino) hexanoate (sIACX), p-nitrophenyl iodoacetate (NPIA), carbonyl-reactive and sulfhydryl-reactive cross-linkers such as 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH), 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide-8 (M2C2H), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), N-hydroxysuccinimidyl-4-azidosalicylic acid (NHs-AsA), N-hydroxysulfosuccinimidyl-4-azidosalicylic acid (sulfo-NHs-AsA), sulfosuccinimidyl-(4-azidosalicylamido)hexanoate (sulfo-NHs-LC-AsA), sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3′-dithiopropionate (sAsD), N-hydroxysuccinimidyl-4-azidobenzoate (HsAB), N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HsAB), N-succinimidyl-6-(4′-azido-2′-nitrophenyl amino)hexanoate (sANPAH), sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate (sulfo-sANPAH), N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOs), sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3′-

dithiopropionate (sAND), N-succinimidyl-4(4-azidophenyl)1,3′ -dithiopropionate (sADP), N-sulfosuccinimidyl(4-azidophenyl)-1,3′ -dithiopropionate (sulfo-sADP), sulfosuccinimidyl 4-(ρ-azidophenyl)butyrate (sulfo-sAPB), sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3′ -dithiopropionate (sAED), sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate (sulfo-sAMCA), p-nitrophenyl diazopyruvate (pNPDP), p-nitrophenyl-2-diazo-3,3,3-trifluoropropionate (PNP-DTP), 1-(ρ-azidosalicylamido)-4-(iodoacetamido)butane (AsIB), N-[4-(ρ-azidosalicylamido)butyl]-3′ -(2′ -pyridyldithio) propionamide (APDP), benzophenone-4-iodoacetamide, p-azidobenzoyl hydrazide (ABH), 4-(ρ-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-aminobenzyoxycarbonyl (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.

[00237] 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^{125}>Ser^{51}), fused via peptidyl linker ($^{60}GG^{61}$) to human interleukin 2 fragment (62-132), fused via peptidyl linker ($^{133}GSGGGS^{138}$) to human interleukin 2 receptor α-chain fragment (139-303), produced in Chinese hamster ovary (CHO) cells, glycosylated; human interleukin 2 (IL-2) (75-133)-peptide [Cys^{125}(51)>Ser]-mutant (1-59), fused via a G_2 peptide linker (60-61) to human interleukin 2 (IL-2) (4-74)-peptide (62-132) and via a GSG_3S peptide linker (133-138) to human interleukin 2 receptor α-chain (IL2R subunit alpha, IL2Rα, 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-1Rα or a protein having at least 98% amino acid sequence identity to IL-1Rα and having the receptor antagonist activity of IL-Rα, and wherein the second fusion partner comprises all or a portion of an immunoglobulin comprising an Fc region, wherein the mucin domain polypeptide linker comprises SEQ ID NO:8 or an amino acid sequence having at least 90% sequence identity to SEQ ID NO:8 and wherein the half-life of the fusion protein is improved as compared to a fusion of the first fusion partner to the second fusion partner in the absence of the mucin domain polypeptide linker.

TABLE 2. Amino acid sequences of interleukins.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:3 recombinant human IL-2 (rhIL-2)	MAPTSSSTKK TQLQLEHLLL DLQMILNGIN NYKNPKLTRM LTFKFYMPKK ATELKHLQCL 60 EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN 120 RWITFCQSII STLT 134
SEQ ID NO:4 Aldesleukin	PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE 60 ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW 120 ITFSQSIIST LT 132
SEQ ID NO:5 IL-2 form	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML TFKFYMPKKA TELKHLQCLE 60 EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120 WITFCQSIIS TLT 133
SEQ ID NO:6 Nemvaleukin alfa	SKNFHLRPRD LISNINVIVL ELKGSETTFM CEYADETATI VEFLNRWITF SQSIISTLTG 60 GSSSTKKTQL QLEHLLLDLQ MILNGINNYK NPKLTRMLTF KFYMPKKATE LKHLQCLEEE 120 LKPLEEVLNL AQGSGGGSEL CDDDPPEIPH ATFKAMAYKE GTMLNCECKR GFRRIKSGSL 180

	YMLCTGNSSH SSWDNQCQCT SSATRNTTKQ VTPQPEEQKE RKTTEMQSPM QPVDQASLPG 240 HCREPPPWEN EATERIYHFV VGQMVYYQCV QGYRALHRGP AESVCKMTHG KTRWTQPQLI 300 CTG 303
SEQ ID NO:7 IL-2 form	MDAMKRGLCC VLLLCGAVFV SARRPSGRKS SKMQAFRIWD VNQKTFYLRN NQLVAGYLQG 60 PNVNLEEKID VVPIEPHALF LGIHGGKMCL SCVKSGDETR LQLEAVNITD LSENRKQDKR 120 FAFIRSDSGP TTSFESAACP GWFLCTAMEA DQPVSLTNMP DEGVMVTKFY FQEDESGSGG 180 ASSESSASSD GPHPVITESR ASSESSASSD GPHPVITESR EPKSSDKTHT CPPCPAPELL 240 GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ 300 YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE PQVYTLPPSR 360 EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PVLDSDGSFF LYSKLTVDKS 420 RWQQGNVFSC SVMHEALHNH YTQKSLSLSP GK 452
SEQ ID NO:8 mucin domain polypeptide	SESSASSDGP HPVITP 16
SEQ ID NO:9 recombinant human IL-4 (rhIL-4)	MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH 60 EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI 120 MREKYSKCSS 130
SEQ ID NO:10 recombinant human IL-7 (rhIL-7)	MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA 60 ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL 120 KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH 153
SEQ ID NO:11 recombinant human IL-15 (rhIL-15)	MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI 60 HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS 115
SEQ ID NO:12 recombinant human IL-21 (rhIL-21)	MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG 60 NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ 120 HLSSRTHGSE DS 132

[0028] In some embodiments, an IL-2 form suitable for use in the invention includes a antibody cytokine engrafted protein comprises a heavy chain variable region (V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L, wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain variable region

(V_H), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (V_L), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L, wherein the IL-2 molecule is a mutein, and wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells. In some embodiments, the IL-2 regimen comprises administration of an antibody described in U.S. Patent Application Publication No. US 2020/0270334 A1, the disclosures of which are incorporated by reference herein. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain variable region (VH), comprising complementarity determining regions HCDR1, HCDR2, HCDR3; a light chain variable region (VL), comprising LCDR1, LCDR2, LCDR3; and an IL-2 molecule or a fragment thereof engrafted into a CDR of the V_H or the V_L, wherein the IL-2 molecule is a mutein, wherein the antibody cytokine engrafted protein preferentially expands T effector cells over regulatory T cells, and wherein the antibody further comprises an IgG class heavy chain and an IgG class light chain selected from the group consisting of: a IgG class light chain comprising SEQ ID NO:39 and a IgG class heavy chain comprising SEQ ID NO:38; a IgG class light chain comprising SEQ ID NO:37 and a IgG class heavy chain comprising SEQ ID NO:29; a IgG class light chain comprising SEQ ID NO:39 and a IgG class heavy chain comprising SEQ ID NO:29; and a IgG class light chain comprising SEQ ID NO:37 and a IgG class heavy chain comprising SEQ ID NO:38.

[0029] In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into HCDR1 of the V_H, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into HCDR2 of the V_H, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into HCDR3 of the V_H, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into LCDR1 of the V_L, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into LCDR2 of the V_L, wherein the IL-2 molecule is a mutein. In some embodiments, an IL-2 molecule or a fragment thereof is engrafted into LCDR3 of the V_L, wherein the IL-2 molecule is a mutein.

[0030] The insertion of the IL-2 molecule can be at or near the N-terminal region of the CDR, in the middle region of the CDR or at or near the C-terminal region of the CDR. In some embodiments, the antibody cytokine engrafted protein comprises an IL-2 molecule incorporated into a CDR, wherein the IL2 sequence does not frameshift the CDR sequence. In some embodiments, the antibody cytokine engrafted protein comprises an IL-2 molecule incorporated into a CDR, wherein the IL-2 sequence replaces all or part of a CDR sequence. The replacement by the IL-2 molecule can be the N-terminal

region of the CDR, in the middle region of the CDR or at or near the C-terminal region the CDR. A replacement by the IL-2 molecule can be as few as one or two amino acids of a CDR sequence, or the entire CDR sequences.

[0031] In some embodiments, an IL-2 molecule is engrafted directly into a CDR without a peptide linker, with no additional amino acids between the CDR sequence and the IL-2 sequence. In some embodiments, an IL-2 molecule is engrafted indirectly into a CDR with a peptide linker, with one or more additional amino acids between the CDR sequence and the IL-2 sequence.

[0032] 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.

[0033] In some embodiments, the antibody cytokine engrafted protein comprises an HCDR1 selected from the group consisting of SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:22 and SEQ ID NO:25. In some embodiments, the antibody cytokine engrafted protein comprises an HCDR1 selected from the group consisting of SEQ ID NO:7, SEQ ID NO:10, SEQ ID NO:13 and SEQ ID NO:16. In some embodiments, the antibody cytokine engrafted protein comprises an HCDR1 selected from the group consisting of HCDR2 selected from the group consisting of SEQ ID NO:17, SEQ ID NO:20, SEQ ID NO:23, and SEQ ID NO:26. In some embodiments, the antibody cytokine engrafted protein comprises an HCDR3 selected from the group consisting of SEQ ID NO:18, SEQ ID NO:21, SEQ ID NO:24, and SEQ ID NO:27. In some embodiments, the antibody cytokine engrafted protein comprises a V_H region comprising the amino acid sequence of SEQ ID NO:28. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain comprising the amino acid sequence of SEQ ID NO:29. In some embodiments, the antibody cytokine engrafted protein comprises a V_L region comprising the amino acid sequence of SEQ ID NO:36. In some embodiments, the antibody cytokine engrafted protein comprises a light chain comprising the amino acid sequence of SEQ ID NO:37. In some embodiments, the antibody cytokine engrafted protein comprises a V_H region comprising the amino acid sequence of SEQ ID NO:28 and a V_L region comprising the amino acid sequence of SEQ ID NO:36. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:29 and a light chain region comprising the amino acid sequence of SEQ ID NO:37. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ

ID NO:29 and a light chain region comprising the amino acid sequence of SEQ ID NO:39. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:38 and a light chain region comprising the amino acid sequence of SEQ ID NO:37. In some embodiments, the antibody cytokine engrafted protein comprises a heavy chain region comprising the amino acid sequence of SEQ ID NO:38 and a light chain region comprising the amino acid sequence of SEQ ID NO:39. In some embodiments, the antibody cytokine engrafted protein comprises IgG.IL2F71A.H1 or IgG.IL2R67A.H1 of U.S. Patent Application Publication No. 2020/0270334 A1, or variants, derivatives, or fragments thereof, or conservative amino acid substitutions thereof, or proteins with at least 80%, at least 90%, at least 95%, or at least 98% sequence identity thereto. In some embodiments, the antibody components of the antibody cytokine engrafted protein described herein comprise immunoglobulin sequences, framework sequences, or CDR sequences of palivizumab. In some embodiments, the antibody cytokine engrafted protein described herein has a longer serum half-life that 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	MYRMQLLSCI ALSLALVTNS APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML	60
IL-2	TFKFYMPKKA TELKHLQCLE EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE	120
	TTFMCEYADE TATIVEFLNR WITFCQSIIS TLT 153	
SEQ ID NO:14	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTAML TFKFYMPKKA TELKHLQCLE	60
IL-2 mutein	EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR	120
	WITFCQSIIS TLT 133	
SEQ ID NO:15	APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML TAKFYMPKKA TELKHLQCLE	60
IL-2 mutein	EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR	120
	WITFCQSIIS TLT 133	
SEQ ID NO:16	GFSLAPTSSS TKKTQLQLEH LLLDLQMILN GINNYKNPKL TAMLTFKFYM PKKATELKHL	60
HCDR1_IL-2	QCLEEELKPL EEVLNLAQSK NFHLRPRDLI SNINVIVLEL KGSETTFMCE YADETATIVE	120
	FLNRWITFCQ SIISTLTSTS GMSVG 145	
SEQ ID NO:17	DIWWDDKKDY NPSLKS 16	
HCDR2		
SEQ ID NO:18	SMITNWYFDV 10	
HCDR3		

SEQ ID NO:19 APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTAML TFKFYMPKKA TELKHLQCLE 60

HCDR1_IL-2 kabat EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE TTFMCEYADE TATIVEFLNR 120

WITFCQSIIS TLTSTSGMSV G 141

SEQ ID NO:20 DIWWDDKKDY NPSLKS 16

HCDR2 kabat

SEQ ID NO:21 SMITNWYFDV 10

HCDR3 kabat

SEQ ID NO:22 GFSLAPTSSS TKKTQLQLEH LLLDLQMILN GINNYKNPKL TAMLTFKFYM PKKATELKHL 60

HCDR1_IL-2 clothia QCLEEELKPL EEVLNLAQSK NFHLRPRDLI SNINVIVLEL KGSETTFMCE YADETATIVE 120

FLNRWITFCQ SIISTLTSTS GM 142

SEQ ID NO:23 WWDDK 5

HCDR2 clothia

SEQ ID NO:24 SMITNWYFDV 10

HCDR3 clothia

SEQ ID NO:25 GFSLAPTSSS TKKTQLQLEH LLLDLQMILN GINNYKNPKL TAMLTFKFYM PKKATELKHL 60

HCDR1_IL-2 IMGT QCLEEELKPL EEVLNLAQSK NFHLRPRDLI SNINVIVLEL KGSETTFMCE YADETATIVE 120

FLNRWITFCQ SIISTLTSTS GMS 143

SEQ ID NO:26 IWWDDKK 7

HCDR2 IMGT

SEQ ID NO:27 ARSMITNWYF DV 12

HCDR3 IMGT

SEQ ID NO:28 QVTLRESGPA LVKPTQTLTL TCTFSGFSLA PTSSSTKKTQ LQLEHLLLDL QMILNGINNY 60

V_H KNPKLTAMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISNINV 120

IVLELKGSET TFMCEYADET ATIVEFLNRW ITFCQSIIST LTSTSGMSVG WIRQPPGKAL 180

EWLADIWWDD KKDYNPSLKS RLTISKDTSK NQVVLKVTNM DPADTATYYC ARSMITNWYF 240

DVWGAGTTVT VSS 253

SEQ ID NO:29 QMILNGINNY KNPKLTAMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR 60

Heavy chain PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW ITFCQSIIST LTSTSGMSVG 120

WIRQPPGKAL EWLADIWWDD KKDYNPSLKS RLTISKDTSK NQVVLKVTNM DPADTATYYC 180

ARSMITNWYF DVWGAGTTVT VSSASTKGPS VFPLAPSSKS TSGGTAALGC LVKDYFPEPV 240

TVSWNSGALT SGVHTFPAVL QSSGLYSLSS VVTVPSSSLG TQTYICNVNH KPSNTKVDKR 300

VEPKSCDKTH TCPPCPAPEL LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV AVSHEDPEVK 360

FNWYVDGVEV HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALAAPIEK 420

TISKAKGQPR EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT 480

PPVLDSDGSF FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK 533

SEQ ID NO:30 KAQLSVGYMH 10

LCDR1 kabat	
SEQ ID NO:31	DTSKLAS 7
LCDR2 kabat	
SEQ ID NO:32	FQGSGYPFT 9
LCDR3 kabat	
SEQ ID NO:33	QLSVGY 6
LCDR1 chothia	
SEQ ID NO:34	DTS 3
LCDR2 chothia	
SEQ ID NO:35	GSGYPF 6
LCDR3 chothia	
SEQ ID NO:36	DIQMTQSPST LSASVGDRVT ITCKAQLSVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR 60
V_L	FSGSGSGTEF TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKLEIK 106
SEQ ID NO:37	DIQMTQSPST LSASVGDRVT ITCKAQLSVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR 60
Light chain	FSGSGSGTEF TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKLEIKRTVA APSVFIFPPS 120
	DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL 180
	SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC 213
SEQ ID NO:38	QVTLRESGPA LVKPTQTLTL TCTFSGFSLA PTSSSTKKTQ LQLEHLLLDL QMILNGINNY 60
Light chain	KNPKLTRMLT AKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISNINV 120
	IVLELKGSET TFMCEYADET ATIVEFLNRW ITFCQSIIST LTSTSGMSVG WIRQPPGKAL 180
	EWLADIWWDD 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
	HNAKTKPREE QYNSTYRVVS VLTVLHQDWL NGKEYKCKVS NKALAAPIEK TISKAKGQPR 480
	EPQVYTLPPS REEMTKNQVS LTCLVKGFYP SDIAVEWESN GQPENNYKTT PPVLDSDGSF 540
	FLYSKLTVDK SRWQQGNVFS CSVMHEALHN HYTQKSLSLS PGK 583
SEQ ID NO:39	DIQMTQSPST LSASVGDRVT ITCKAQLSVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR 60
Light chain	FSGSGSGTEF TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKLEIKRTVA APSVFIFPPS 120
	DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL 180
	SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC 213

[0034] The term "IL-4" (also referred to herein as "IL4") refers to the cytokine known as interleukin 4, which is produced by Th2 T cells and by eosinophils, basophils, and mast cells. IL-4 regulates the differentiation of naïve helper T cells (Th0 cells) to Th2 T cells. Steinke and Borish, *Respir. Res.* **2001,** *2,* 66-70. Upon activation by IL-4, Th2 T cells subsequently produce additional IL-4 in a positive feedback

loop. IL-4 also stimulates B cell proliferation and class II MHC expression, and induces class switching to IgE and IgG_1 expression from B cells. Recombinant human IL-4 suitable for use in the invention is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-211) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15 recombinant protein, Cat. No. Gibco CTP0043). The amino acid sequence of recombinant human IL-4 suitable for use in the invention is given in Table 2 (SEQ ID NO:9).

[0035] 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).

[0036] The term "IL-15" (also referred to herein as "IL15") refers to the T cell growth factor known as interleukin-15, and includes all forms of IL-2 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-15 is described, *e.g.*, in Fehniger and Caligiuri, *Blood* **2001,** *97,* 14-32, the disclosure of which is incorporated by reference herein. IL-15 shares β and γ signaling receptor subunits with IL-2. Recombinant human IL-15 is a single, non-glycosylated polypeptide chain containing 114 amino acids (and an N-terminal methionine) with a molecular mass of 12.8 kDa. Recombinant human IL-15 is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-230-b) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15 recombinant protein, Cat. No. 34-8159-82). The amino acid sequence of recombinant human IL-15 suitable for use in the invention is given in Table 2 (SEQ ID NO:11).

[0037] The term "IL-21" (also referred to herein as "IL21") refers to the pleiotropic cytokine protein known as interleukin-21, and includes all forms of IL-21 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-21 is described, *e.g.*, in Spolski and Leonard, *Nat. Rev. Drug. Disc.* **2014,** *13,* 379-95, the disclosure of which is

incorporated by reference herein. IL-21 is primarily produced by natural killer T cells and activated human $CD4^+$ T cells. Recombinant human IL-21 is a single, non-glycosylated polypeptide chain containing 132 amino acids with a molecular mass of 15.4 kDa. Recombinant human IL-21 is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-408-b) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-21 recombinant protein, Cat. No. 14-8219-80). The amino acid sequence of recombinant human IL-21 suitable for use in the invention is given in Table 2 (SEQ ID NO:21).

[0038] 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.

[0039] 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.

[0040] 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.

[0041] 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.

[0042] In some embodiments, the invention includes a method of treating a cancer with a population of TILs, wherein a patient is pre-treated with non-myeloablative chemotherapy prior to an infusion of TILs according to the invention. In some embodiments, the population of TILs may be provided wherein a patient is pre-treated with nonmyeloablative chemotherapy prior to an infusion of TILs according to the present invention. In some embodiments, the non-myeloablative chemotherapy is cyclophosphamide 60 mg/kg/d for 2 days (days 27 and 26 prior to TIL infusion) and fludarabine 25 mg/m2/d for 5 days (days 27 to 23 prior to TIL infusion). In some embodiments, after non-myeloablative chemotherapy and TIL infusion (at day 0) according to the invention, the patient receives an intravenous infusion of IL-2 intravenously at 720,000 IU/kg every 8 hours to physiologic tolerance.

[0043] 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.

[0044] 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.

[0045] 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.

[0046] The terms "non-myeloablative chemotherapy," "non-myeloablative lymphodepletion," "NMALD," "NMA LD," "NMA-LD," and any variants of the foregoing, are used interchangeably to indicate a chemotherapeutic regimen designed to deplete the patient's lymphoid immune cells while avoiding depletion of the patient's myeloid immune cells. Typically, the patient receives a course of non-myeloablative chemotherapy prior to the administration of tumor infiltrating lymphocytes to the patient as described herein.

[0047] 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).

[0048] 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.

[0049] 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.

[0050] By "tumor infiltrating lymphocytes" or "TILs" herein is meant a population of cells originally obtained as white blood cells that have left the bloodstream of a subject and migrated into a tumor. TILs include, but are not limited to, $CD8^+$ cytotoxic T cells (lymphocytes), Th1 and Th17 $CD4^+$ T cells, natural killer cells, dendritic cells and M1 macrophages. TILs include both primary and secondary TILs. "Primary

TILs" are those that are obtained from patient tissue samples as outlined herein (sometimes referred to as "freshly harvested"), and "secondary TILs" are any TIL cell populations that have been expanded or proliferated as discussed herein, including, but not limited to bulk TILs, expanded TILs ("REP TILs") as well as "reREP TILs" as discussed herein. reREP TILs can include for example second expansion TILs or second additional expansion TILs (such as, for example, those described in Step D of Figure 8, including TILs referred to as reREP TILs).

[0051] TILs can generally be defined either biochemically, using cell surface markers, or functionally, by their ability to infiltrate tumors and effect treatment. TILs can be generally categorized by expressing one or more of the following biomarkers: CD4, CD8, TCR αβ, CD27, CD28, CD56, CCR7, CD45Ra, CD95, PD-1, and CD25. Additionally, and alternatively, TILs can be functionally defined by their ability to infiltrate solid tumors upon reintroduction into a patient. TILs may further be characterized by potency – for example, TILs may be considered potent if, for example, interferon (IFN) release is greater than about 50 pg/mL, greater than about 100 pg/mL, greater than about 150 pg/mL, or greater than about 200 pg/mL. TILs may be considered potent if, for example, interferon (IFNγ) 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.

[0052] 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.

[0053] 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.

[0054] 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.

[0055] 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.

[0056] 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."

[0057] The terms "antibody" and its plural form "antibodies" refer to whole immunoglobulins and any antigen-binding fragment ("antigen-binding portion") or single chains thereof. An "antibody" further refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen-binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions of an antibody may be further subdivided into regions of hypervariability, which are referred to as complementarity determining regions (CDR) or hypervariable regions (HVR), and which can be interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen epitope or epitopes. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

[0058] 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.

[0059] 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.

[0060] The terms "antigen-binding portion" or "antigen-binding fragment" of an antibody (or simply "antibody portion" or "fragment"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a domain antibody (dAb) fragment (Ward, *et al., Nature,* **1989,** *341,* 544-546), which may consist of a V_H or a V_L domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules known as single chain Fv (scFv); see, *e.g.*, Bird, *et al., Science* **1988,** *242,* 423-426; and Huston, *et al., Proc. Natl. Acad. Sci. USA* **1988,** *85,* 5879-5883). Such scFv antibodies are also intended to be encompassed within the terms "antigen-binding portion" or "antigen-binding fragment" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. In some embodiments, a scFv protein domain comprises a V_H portion and a V_L portion. A scFv molecule is denoted as either V_L-L-V_H if the V_L domain is the N-terminal part of the scFv molecule, or as V_H-L-V_L if the V_H domain is the N-terminal part of the scFv molecule. Methods for making scFv molecules and designing suitable peptide

linkers are described in U.S. Pat. No. 4,704,692, U.S. Pat. No. 4,946,778, R. Raag and M. Whitlow, "Single Chain Fvs." FASEB Vol 9:73-80 (1995) and R. E. Bird and B. W. Walker, Single Chain Antibody Variable Regions, TIBTECH, Vol 9: 132-137 (1991), the disclosures of which are incorporated by reference herein.

[0061] 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.

[0062] 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.

[0063] The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (such as a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further below), (b) antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the V_H and V_L regions of the recombinant antibodies are sequences that, while derived

from and related to human germline V_H and V_L sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[0064] As used herein, "isotype" refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by the heavy chain constant region genes.

[0065] 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."

[0066] 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.

[0067] 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.

[0068] 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.

[0069] A "diabody" is a small antibody fragment with two antigen-binding sites. The fragments comprises a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L or V_L-V_H). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, *e.g.*, European Patent No. EP 404,097, International Patent Publication No. WO 93/11161; and Bolliger, *et al.*, *Proc. Natl. Acad. Sci. USA* **1993,** *90,* 6444-6448.

[0070] The term "glycosylation" refers to a modified derivative of an antibody. An aglycoslated antibody lacks glycosylation. Glycosylation can be altered to, for example, increase the affinity of the antibody for antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Aglycosylation may increase the affinity of the antibody for antigen, as described in U.S. Patent Nos. 5,714,350 and 6,350,861. Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased

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

[0071] "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_1-C_{10})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.

[0072] 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.

II. Gene-Editing Processes

A. Overview: TIL Expansion + Gene-Editing

[0073] Embodiments of the present invention are directed to methods for expanding TIL populations, the methods comprising one or more steps of gene-editing at least a portion of the TILs in order to enhance their therapeutic effect. As used herein, "gene-editing," "gene editing," and "genome editing" refer to a type of genetic modification in which DNA is permanently modified in the genome of a cell, *e.g.*, DNA is inserted, deleted, modified or replaced within the cell's genome. In some embodiments, gene-editing causes the expression of a DNA sequence to be silenced (sometimes referred to as a gene knockout) or inhibited/reduced (sometimes referred to as a gene knockdown). In accordance with embodiments of the present invention, gene-editing technology is used to enhance the effectiveness of a therapeutic population of TILs.

[0074] A method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein, wherein the method further comprises gene-editing at least a portion of the TILs. According to additional embodiments, a method for expanding TILs into a therapeutic population of TILs is carried out in accordance with any embodiment of the methods described in WO 2018/081473 A1, WO 2018/129332 A1, or WO 2018/182817 A1, which are incorporated by reference herein in their entireties, wherein the method further comprises gene-editing at least a portion of the TILs. Thus, an embodiment of the present invention provides a therapeutic population of TILs that has been expanded in accordance with any embodiment described herein, wherein at least a portion of the therapeutic population has been gene-edited, *e.g.,* at least a portion of the therapeutic population of TILs that is transferred to the infusion bag is permanently gene-edited.

B. Gene-Editing During TIL Expansion

[0075] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;

(c) activating the second population of TILs using anti-CD3 and anti-CD28 beads or antibodies for 1- 7 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and

(e) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0076] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor tissue to produce a tumor digest;

(c) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;

(d) activating the second population of TILs using anti-CD3 and anti-CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(e) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and

(f) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0077] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;

(c) activating the second population of TILs using anti-CD3 and anti-CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(e) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 1-7 days, to produce a culture of a fifth population of TILs; and

(f) splitting the culture of the fifth population of TILs into a plurality of subcultures, culturing each of the plurality of subcultures in a third cell culture medium comprising IL-2 for about 3-7 days, and combining the plurality of subcultures to provide an expanded number of TILs.

[0078] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor tissue to produce a tumor digest;

(c) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;

(d) activating the second population of TILs using anti-CD3 and anti-CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(e) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(f) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 1-7 days, to produce a culture of a fifth population of TILs; and

(g) splitting the culture of the fifth population of TILs into a plurality of subcultures, culturing each of the plurality of subcultures in a third cell culture medium comprising IL-2 for about 3-7 days, and combining the plurality of subcultures to provide an expanded number of TILs.

[0079] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained by digesting in an enzyme media a tumor tissue resected from a subject or patient to produce a tumor digest in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(c) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[0080] In some embodiments, the method comprises the step of culturing or initial expansion of the first population of TILs comprises culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3 days followed by in a cell culture medium comprising IL-2 and OKT-3 for 2-6 days.

[0081] In some embodiments, the method comprises the step of culturing or rapid second expansion of the third population of TILs is performed by culturing the third population of TILs in the second cell culture medium for a first period of about 1-7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3-7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0082] In some embodiments, the step of culturing the first population of TILs is performed for about 3-9 days. In some embodiments, the step of culturing the first population of TILs is performed for about 3-9 days, about 3-8 days, about 4-8 days, about 5-8 days, about 6-8 days, about 7-8 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 3-6 days, about 4-6 days, about 5-6 days, about 3-5 days, about 4-5 days, about 3-4 days. In some embodiments, the step of culturing the first population of TILs is performed for about 3 days. In some embodiments, the step of culturing the first population of

TILs is performed for about 4 days. In some embodiments, the step of culturing the first population of TILs is performed for about 5 days. In some embodiments, the step of culturing the first population of TILs is performed for about 6 days. In some embodiments, the step of culturing the first population of TILs is performed for about 7 days. In some embodiments, the step of culturing the first population of TILs is performed for about 8 days. In some embodiments, the step of culturing the first population of TILs is performed for about 9 days.

[0083] In some embodiments, the step of activating the second population of TILs is performed for about 1-7 days. In some embodiments, the step of activating the second population of TILs is performed for about 1-7 days, about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4, days, about 2-4, days, about 3-4, days, about 1-3 days, about 2-3 days, about 1-2 days. 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. In some embodiments, the step of activating the second population of TILs is performed for about 4 days. In some embodiments, the step of activating the second population of TILs is performed for about 5 days. In some embodiments, the step of activating the second population of TILs is performed for about 6 days. In some embodiments, the step of activating the second population of TILs is performed for about 7 days.

[0084] In some embodiments, the step of culturing the fourth population of TILs is performed for about 5-15 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 5-15 days, about 6-15 days, about 7-15 days, about 8-15 days, about 9-15 days, about 10-15 days, about 11-15 days, about 12-15 days, about 13-15 days, about 14-15 days, about 5-14 days, about 6-14 days, about 7-14 days, about 8-14 days, about 9-14 days, about 10-14 days, about 11-14 days, about 12-14 days, about 13-14 days, about 5-13 days, about 6-13 days, about 7-13 days, about 8-13 days, about 9-13 days, about 10-13 days, about 11-13 days, about 12-13 days, about 5-12 days, about 6-12 days, about 7-12 days, about 8-12 days, about 9-12 days, about 10-12 days, about 11-12 days, about 5-11 days, 6-11 days, 7-11 days, about 8-11 days, about 9-11 days, about 10-11 days, about 5-10 days, 6-10 days, 7-10 days, about 8-10 days, about 9-10 days, about 5-9 days, 6-9 days, 7-9 days, about 8-9 days, about 5-8 days, about 6-8 days, 7-8 days, about 5-7 days, about 6-7 days, about 5-6 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 5 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 6 days. In some embodiments,

the step of culturing the fourth population of TILs is performed for about 7 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 8 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 9 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 10 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 11 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 12 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 13 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 14 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 15 days.

[0085] 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.

[0086] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0087] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0088] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0089] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0090] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at

the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0091] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0092] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0093] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0094] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0095] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at

the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0096] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0097] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0098] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[0099] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00100] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3

days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00101] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00102] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00103] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00104] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00105] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4

days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00106] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00107] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00108] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00109] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00110] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5

days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00111] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00112] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00113] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00114] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00115] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6

days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00116] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00117] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00118] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00119] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00120] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7

days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00121] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00122] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00123] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(e), (a)-(f), or (a)-(g) outlined in the methods above, or before or after any of steps (a)-(e), (a)-(f), or (a)-(g) outlined in the methods above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00124] It should be noted that alternative embodiments of the expansion process may differ from the methods shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(e), (a)-(f), or (a)-(g), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing

process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00125] According to some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.,* they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00126] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 and OKT-3 for about 3- 9 days to produce a second population of TILs;

(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(d) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[00127] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor tissue to produce a tumor digest;

(c) culturing the first population of TILs in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(d) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(e) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[00128] In some embodiments, the step of culturing the first population of TILs is performed for about 3-9 days. In some embodiments, the step of culturing the first population of TILs is performed for about 3-9 days, about 3-8 days, about 4-8 days, about 5-8 days, about 6-8 days, about 7-8 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 3-6 days, about 4-6 days, about 5-6 days, about 3-5 days, about 4-5 days, about 3-4 days. In some embodiments, the step of culturing the first population of TILs is performed for about 3 days. In some embodiments, the step of culturing the first population of TILs is performed for about 4 days. In some embodiments, the step of culturing the first population of TILs 5 days. In some embodiments, the step of culturing the first population of TILs is performed for about 6 days. In some embodiments, the step of culturing the first population of TILs is performed for about 7 days. In some embodiments, the step of culturing the first population of TILs is performed for about 8 days. In some embodiments, the step of culturing the first population of TILs is performed for about 9 days.

[00129] In some embodiments, the step of culturing the third population of TILs is performed for about 5-15 days. In some embodiments, the step of culturing the third population of TILs is performed for about 5-15 days, about 6-15 days, about 7-15 days, about 8-15 days, about 9-15 days, about 10-15 days, about 11-15 days, about 12-15 days, about 13-15 days, about 14-15 days, about 5-14 days, about 6-14 days, about 7-14 days, about 8-14 days, about 9-14 days, about 10-14 days, about 11-14 days, about 12-14 days, about 13-14 days, about 5-13 days, about 6-13 days, about 7-13 days, about 8-13 days, about 9-13 days, about 10-13 days, about 11-13 days, about 12-13 days, about 5-12 days, about 6-12 days, about 7-12 days, about 8-12 days, about 9-12 days, about 10-12 days, about 11-12 days, about 5-11 days, 6-11 days, 7-11 days, about 8-11 days, about 9-11 days, about 10-11 days, about 5-10 days, 6-10 days, 7-10 days, about 8-10 days, about 9-10 days, about 5-9 days, 6-9 days, 7-9 days, about 8-9 days, about 5-8 days, about 6-8 days, 7-8 days, about 5-7 days, about 6-7 days, about 5-6 days. In some embodiments, the step of culturing the third population of TILs is performed for about 5 days. In some embodiments, the step of culturing the third population of TILs is performed for about 6 days. In some embodiments, the step of culturing the third population of TILs is performed for about 7 days. In some embodiments, the step of culturing the third population of TILs is performed for about 8 days. In some embodiments, the step of culturing the third population of TILs is performed for about 9 days. In some

embodiments, the step of culturing the third population of TILs is performed for about 10 days. In some embodiments, the step of culturing the third population of TILs is performed for about 11 days. In some embodiments, the step of culturing the third population of TILs is performed for about 12 days. In some embodiments, the step of culturing the third population of TILs is performed for about 13 days. In some embodiments, the step of culturing the third population of TILs is performed for about 14 days. In some embodiments, the step of culturing the third population of TILs is performed for about 15 days.

[00130] 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.

[00131] In some embodiments, the step of culturing the third population of TILs is performed by culturing the third population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00132] In some embodiments, the step of culturing the third population of TILs is performed by culturing the third population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00133] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(d) or (a)-(e) outlined in the method above, or before or after any of steps (a)-(d) or (a)-(e) outlined in the method above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00134] It should be noted that alternative embodiments of the expansion process may differ from the method shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(d) or (a)-(e), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00135] According to some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.*, they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00136] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs;

(d) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 1-7 days, to produce a culture of a fourth population of TILs; and

(e) splitting the culture of the fourth population of TILs into a plurality of subcultures, culturing each of the plurality of subcultures in a third cell culture medium comprising IL-2 for about 3-7 days, and combining the plurality of subcultures to provide a fifth population of TILs comprising an expanded number of TILs.

[00137] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor tissue to produce a tumor digest;

(c) culturing the first population of TILs in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;

(d) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs;

(e) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 1-7 days, to produce a culture of a fourth population of TILs; and

(f) splitting the culture of the fourth population of TILs into a plurality of subcultures, culturing each of the plurality of subcultures in a third cell culture medium comprising IL-2 for about 3-7

days, and combining the plurality of subcultures to provide a fifth population of TILs comprising an expanded number of TILs.

[00138] In some embodiments, the step of culturing the first population of TILs is performed for about 3-9 days. In some embodiments, the step of culturing the first population of TILs is performed for about 3-9 days, about 3-8 days, about 4-8 days, about 5-8 days, about 6-8 days, about 7-8 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 3-6 days, about 4-6 days, about 5-6 days, about 3-5 days, about 4-5 days, about 3-4 days. In some embodiments, the step of culturing the first population of TILs is performed for about 3 days. In some embodiments, the step of culturing the first population of TILs is performed for about 4 days. In some embodiments, the step of culturing the first population of TILs is performed for about 5 days. In some embodiments, the step of culturing the first population of TILs is performed for about 6 days. In some embodiments, the step of culturing the first population of TILs is performed for about 7 days. In some embodiments, the step of culturing the first population of TILs is performed for about 8 days. In some embodiments, the step of culturing the first population of TILs is performed for about 9 days.

[00139] In some embodiments, the step of culturing the third population of TILs is performed for about 1-7 days. In some embodiments, the step of culturing the third population of TILs is performed for about 1-7 days, about 2-7 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4 days, about 2-4 days, about 3-4 days, about 1-3 days, about 2-3 days, about 1-2 days. In some embodiments, the step of culturing the third population of TILs is performed for about 1 day. In some embodiments, the step of culturing the third population of TILs is performed for about 2 days. In some embodiments, the step of culturing the third population of TILs is performed for about 3 days. In some embodiments, the step of culturing the third population of TILs is performed for about 4 days. In some embodiments, the step of culturing the third population of TILs is performed for about 5 days. In some embodiments, the step of culturing the third population of TILs is performed for about 6 days. In some embodiments, the step of culturing the third population of TILs is performed for about 7 days.

[00140] In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 3-6 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 3-6 days, about 4-6 days, about 5-6 days, about 3-5 days, about 4-5 days, about 3-4 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for

about 3 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 4 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 5 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 6 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 7 days.

[00141] 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.

[00142] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(e) or (a)-(f) outlined in the methods above, or before or after any of steps (a)-(e) or (a)-(f) outlined in the methods above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a

portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00143] It should be noted that alternative embodiments of the expansion process may differ from the methods shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(e) or (a)-(f), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00144] According to some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.,* they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00145] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3 days to produce a second population of TILs;

(c) culturing the second population of TILs in a second cell culture medium comprising IL-2 and OKT-3 for 2-4 days to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and

(e) culturing the fourth population of TILs in a third cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[00146] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor tissue to produce a tumor digest;

(c) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3 days to produce a second population of TILs;

(d) culturing the second population of TILs in a second cell culture medium comprising IL-2 and OKT-3 for 2-4 days to produce a third population of TILs;

(e) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and

(f) culturing the fourth population of TILs in a third cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

[00147] In some embodiments, the step of culturing the second population of TILs is performed for about 2-4 days. In some embodiments, the step of culturing the third population of TILs is performed for about 2-4 days, about 3-4 days, about 2-3 days. In some embodiments, the step of culturing the second population of TILs is performed for about 2 days. In some embodiments, the step of culturing the second population of TILs is performed for about 3 days. In some embodiments, the step of culturing the second population of TILs is performed for about 4 days.

[00148] In some embodiments, the step of culturing the fourth population of TILs is performed for about 5-15 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 5-15 days, about 6-15 days, about 7-15 days, about 8-15 days, about 9-15 days, about 10-15 days, about 11-15 days, about 12-15 days, about 13-15 days, about 14-15 days, about 5-14 days, about 6-14 days, about 7-14 days, about 8-14 days, about 9-14 days, about 10-14 days, about 11-14 days, about 12-14 days, about 13-14 days, about 5-13 days, about 6-13 days, about 7-13 days, about 8-13 days, about 9-13 days, about 10-13 days, about 11-13 days, about 12-13 days, about 5-12 days, about 6-12 days, about 7-12 days, about 8-12 days, about 9-12 days, about 10-12 days, about 11-12 days, about 5-11 days, 6-11 days, 7-11 days, about 8-11 days, about 9-11 days, about 10-11 days, about 5-10 days, 6-10 days, 7-10 days, about 8-10 days, about 9-10 days, about 5-9 days, 6-9 days, 7-9 days, about 8-9 days, about 5-8 days, about 6-8 days, 7-8 days, about 5-7 days, about 6-7 days, about 5-6 days. In some

embodiments, the step of culturing the fourth population of TILs is performed for about 5 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 6 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 7 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 8 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 9 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 10 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 11 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 12 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 13 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 14 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 15 days.

[00149] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00150] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00151] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00152] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00153] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00154] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00155] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00156] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00157] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00158] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00159] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00160] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00161] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00162] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00163] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00164] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00165] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00166] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00167] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00168] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00169] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00170] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00171] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00172] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00173] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00174] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00175] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00176] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00177] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00178] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00179] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00180] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00181] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00182] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00183] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00184] 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.

[00185] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(f) or (a)-(g) outlined

in the methods above, or before or after any of steps (a)-(f) or (a)-(g) outlined in the methods above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00186] It should be noted that alternative embodiments of the expansion process may differ from the methods shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(f) or (a)-(g), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00187] According to some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.,* they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00188] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3 days to produce a second population of TILs;

(c) culturing the second population of TILs in a second cell culture medium comprising IL-2 and OKT-3 for 2-4 days to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(e) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 1-7 days, to produce a culture of a fourth population of TILs; and

(f) splitting the culture of the fourth population of TILs into a plurality of subcultures, culturing each of the plurality of subcultures in a third cell culture medium comprising IL-2 for about 3-7 days, and combining the plurality of subcultures to provide a fifth population of TILs comprising an expanded number of TILs.

[00189] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor tissue to produce a tumor digest;

(c) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3 days to produce a second population of TILs;

(d) culturing the second population of TILs in a second cell culture medium comprising IL-2 and OKT-3 for 2-4 days to produce a third population of TILs;

(e) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(f) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 1-7 days, to produce a culture of a fifth population of TILs; and

(g) splitting the culture of the fifth population of TILs into a plurality of subcultures, culturing each of the plurality of subcultures in a third cell culture medium comprising IL-2 for about 3-7 days, and combining the plurality of subcultures to provide a fifth population of TILs comprising an expanded number of TILs.

[00190] In some embodiments, the step of culturing the second population of TILs is performed for about 2-4 days. In some embodiments, the step of culturing the third population of TILs is performed for about 2-4 days, about 3-4 days, about 2-3 days. In some embodiments, the step of culturing the second population of TILs is performed for about 2 days. In some embodiments, the step of culturing the second

population of TILs is performed for about 3 days. In some embodiments, the step of culturing the second population of TILs is performed for about 4 days.

[00191] In some embodiments, the step of culturing the fourth population of TILs is performed for about 1-7 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 1-7 days, about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4, days, about 2-4, days, about 3-4, days, about 1-3 days, about 2-3 days, about 1-2 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 1 day. In some embodiments, the step of culturing the fourth population of TILs is performed for about 2 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 3 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 4 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 5 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 6 days. In some embodiments, the step of culturing the fourth population of TILs is performed for about 7 days.

[00192] In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 3-6 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 3-6 days, about 4-6 days, about 5-6 days, about 3-5 days, about 4-5 days, about 3-4 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 3 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 4 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 5 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 6 days. In some embodiments, the step of culturing each of the plurality of subcultures is performed for about 7 days.

[00193] 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.

[00194] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(f) or (a)-(g) outlined in the methods above, or before or after any of steps (a)-(f) or (a)-(g) outlined in the methods above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00195] It should be noted that alternative embodiments of the expansion process may differ from the methods shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(f) or (a)-(g), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00196] According to some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.,* they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00197] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 9 days;

(c) activating the second population of TILs using anti-CD3 and anti-CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(e) performing a rapid second expansion of the fourth population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[00198] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 9 days;

(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(d) performing a rapid second expansion of the third population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[00199] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor fragments to produce a tumor digest;

(c) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), where the priming first expansion occurs for a period of about 1 to 9 days;

(d) activating the second population of TILs using anti-CD3 and anti-CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(e) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(f) performing a rapid second expansion of the fourth population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[00200] In some embodiments, a method for preparing expanded tumor infiltrating lymphocytes (TILs) comprises:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;

(b) digesting in an enzyme media the tumor fragments to produce a tumor digest;

(c) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), where the priming first expansion occurs for a period of about 1 to 9 days;

(d) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(e) performing a rapid second expansion of the third population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

[00201] In some embodiments, the initial expansion is performed for about 3-9 days. In some embodiments, the initial expansion is performed for about 1-9 days, 2-9 days, 3-9 days, about 4-9 days, about 5-9 days, about 6-9 days, about 7-9 days, about 8-9 days, about 1-8 days, about 2-8 days, about 3-8 days, about 4-8 days, about 5-8 days, about 6-8 days, about 7-8 days, about 1-7 days, about 2-7 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4 days, about 2-4 days, about 3-4 days, about 1-3 days, about 2-3 days, or about 1-2 days. In some embodiments, the initial expansion is performed for about 1 day. In some embodiments, the initial expansion is performed for about 2 days. In some embodiments, the initial expansion is performed for about 3 days. In some embodiments, the initial expansion is performed for about 4 days. In some embodiments, the initial expansion is performed for about 5 days. In some embodiments, the initial expansion is performed for about 6 days. In some embodiments, the initial expansion is performed for about 7 days. In some embodiments, the initial expansion is performed for about 8 days. In some embodiments, the initial expansion is performed for about 9 days.

[00202] In some embodiments, the step of activating the second population of TILs is performed for about 1-7 days. In some embodiments, the step of activating the second population of TILs is performed for about 1-7 days, about 2-7 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days,

about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4, days, about 2-4, days, about 3-4, days, about 1-3 days, about 2-3 days, or about 1-2 days. 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. In some embodiments, the step of activating the second population of TILs is performed for about 4 days. In some embodiments, the step of activating the second population of TILs is performed for about 5 days. In some embodiments, the step of activating the second population of TILs is performed for about 6 days. In some embodiments, the step of activating the second population of TILs is performed for about 7 days.

[00203] In some embodiments, the rapid second expansion is performed for about 5-15 days. In some embodiments, the rapid second expansion is performed for about 5-15 days, about 6-15 days, about 7-15 days, about 8-15 days, about 9-15 days, about 10-15 days, about 11-15 days, about 12-15 days, about 13-15 days, about 14-15 days, about 5-14 days, about 6-14 days, about 7-14 days, about 8-14 days, about 9-14 days, about 10-14 days, about 11-14 days, about 12-14 days, about 13-14 days, about 5-13 days, about 6-13 days, about 7-13 days, about 8-13 days, about 9-13 days, about 10-13 days, about 11-13 days, about 12-13 days, about 5-12 days, about 6-12 days, about 7-12 days, about 8-12 days, about 9-12 days, about 10-12 days, about 11-12 days, about 5-11 days, 6-11 days, 7-11 days, about 8-11 days, about 9-11 days, about 10-11 days, about 5-10 days, 6-10 days, 7-10 days, about 8-10 days, about 9-10 days, about 5-9 days, 6-9 days, 7-9 days, about 8-9 days, about 5-8 days, about 6-8 days, 7-8 days, about 5-7 days, about 6-7 days, about 5-6 days. In some embodiments, the rapid second expansion is performed for about 5 days. In some embodiments, the rapid second expansion is performed for about 6 days. In some embodiments, the rapid second expansion is performed for about 7 days. In some embodiments, the rapid second expansion is performed for about 8 days. In some embodiments, the rapid second expansion is performed for about 9 days. In some embodiments, the rapid second expansion is performed for about 10 days. In some embodiments, the rapid second expansion is performed for about 11 days. In some embodiments, the rapid second expansion is performed for about 12 days. In some embodiments, the rapid second expansion is performed for about 13 days. In some embodiments, the rapid second expansion is performed for about 14 days. In some embodiments, the rapid second expansion is performed for about 15 days.

[00204] 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.

[00205] In some embodiments, the rapid second expansion is performed by culturing the third population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00206] In some embodiments, the rapid second expansion is performed by culturing the third population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00207] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(e) or (a)-(f) outlined in the methods above, or before or after any of steps (a)-(e) or (a)-(f) outlined in the methods above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00208] It should be noted that alternative embodiments of the expansion process may differ from the methods shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(e) or (a)-(f), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00209] According to some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.,* they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00210] In some embodiments, a method for expanding tumor infiltrating lymphocytes into a therapeutic population of TILs comprises:

(a) obtaining and/or receiving a first population of TILs from a sample of tumor tissue produced by surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining tumor tissue from a patient or subject;

(b) adding the tumor tissue into a closed system and performing a first expansion by culturing the first population of TILs in a first 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 3-9 days to obtain the second population of TILs;

(c) activating the second population of TILs using CD3 and CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(e) performing a second expansion by culturing the fourth population of TILs in a second cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fifth population of TILs, wherein the second expansion is performed for about 5-15 days to obtain the third population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, wherein the fifth population of TILs is a therapeutic population of TILs; and

(f) harvesting the therapeutic population of TILs obtained from step (e), wherein each of steps (b) to (f) is performed in a closed, sterile system, and wherein the transition from step (b) to step (c), the transition from step (c) to step (d), the transition from step (d) to step (e) and/or the transition from step (e) to step (f) occurs without opening the system.

[00211] In some embodiments, a method for expanding tumor infiltrating lymphocytes into a therapeutic population of TILs comprises:

(a) obtaining and/or receiving a first population of TILs from a sample of tumor tissue produced by surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining tumor tissue from a patient or subject;

(b) digesting the sample of tumor tissue or tumor fragments in an enzymatic media to produce a tumor digest;

(c) adding the tumor tissue into a closed system and performing a first expansion by culturing the first population of TILs in a first 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 3-9 days to obtain the second population of TILs;

(d) activating the second population of TILs using CD3 and CD28 beads or antibodies for 1-7 days, to produce a third population of TILs;

(e) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;

(f) performing a second expansion by culturing the fourth population of TILs in a second cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fifth population of TILs, wherein the second expansion is performed for about 5-15 days to obtain the third population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, wherein the fifth population of TILs is a therapeutic population of TILs; and

(g) harvesting the therapeutic population of TILs obtained from step (e), wherein each of steps (c) to (g) is performed in a closed, sterile system, and wherein the transition from step (c) to step (d), the transition from step (d) to step (e), the transition from step (e) to step (f) and/or the transition from step (f) to step (g) occurs without opening the system.

[00212] In some embodiments, the first expansion is performed for about 3-9 days. In some embodiments, the first expansion is performed for about 3-9 days, about 3-8 days, about 3-7 days, about 3-6 days, about 3-5 days, about 3-4 days, about 4-9 days, about 4-8 days, about 5-9 days, about 5-8 days, about 6-9 days, about 6-8 days, about 7-9 days, about 7-8 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 3-6 days, about 4-6 days, about 5-6 days, about 3-5 days, about 4-5 days, about 3-4 days. In some embodiments, the first expansion is performed for about 3 days. In some embodiments, the first expansion is performed for about 4 days. In some embodiments, the first expansion is performed for about 5 days. In some embodiments, the first expansion is performed for about 6 days. In some embodiments, the first expansion is performed for about 7 days. In some embodiments, the first expansion is performed for about 8 days. In some embodiments, the first expansion is performed for about 9 days.

[00213] In some embodiments, the step of activating the second population of TILs is performed for about 1-7 days. In some embodiments, the step of activating the second population of TILs is performed for about 1-7 days, about 2-7 days, about 3-7 days, 4-7 days, about 5-7 days, about 6-7 days, about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4, days, about 2-4, days, about 3-4, days, about 1-3 days, about 2-3 days, about 1-2 days. 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. In some embodiments, the step of activating the second population of TILs is performed for about 4 days. In some embodiments, the step of activating the second population of TILs is performed for about 5 days. In some embodiments, the step of activating the second population of TILs is performed for about 6 days. In some embodiments, the step of activating the second population of TILs is performed for about 7 days.

[00214] In some embodiments, the second expansion is performed for about 5-15 days. In some embodiments, the second expansion is performed for about 5-15 days, about 6-15 days, about 7-15 days, about 8-15 days, about 9-15 days, about 10-15 days, about 11-15 days, about 12-15 days, about 13-15 days, about 14-15 days, about 5-14 days, about 6-14 days, about 7-14 days, about 8-14 days, about 9-14 days, about 10-14 days, about 11-14 days, about 12-14 days, about 13-14 days, about 5-13 days, about 6-13 days, about 7-13 days, about 8-13 days, about 9-13 days, about 10-13 days, about 11-13 days, about 12-13 days, about 5-12 days, about 6-12 days, about 7-12 days, about 8-12 days, about 9-12 days, about 10-12 days, about 11-12 days, about 5-11 days, 6-11 days, 7-11 days, about 8-11 days, about 9-11 days, about 10-11 days, about 5-10 days, 6-10 days, 7-10 days, about 8-10 days, about 9-10 days, about 5-9 days, 6-9 days, 7-9 days, about 8-9 days, about 5-8 days, about 6-8 days, 7-8 days, about 5-7 days, about 6-7 days, about 5-6 days. In some embodiments, the second expansion is performed for about 5 days. In some embodiments, the second expansion is performed for about 6 days. In some embodiments, the second expansion is performed for about 7 days. In some embodiments, the second expansion is performed for about 8 days. In some embodiments, the second expansion is performed for about 9 days. In some embodiments, the second expansion is performed for about 10 days. In some embodiments, the second expansion is performed for about 11 days. In some embodiments, the second expansion is performed for about 12 days. In some embodiments, the second expansion is performed

for about 13 days. In some embodiments, the second expansion is performed for about 14 days. In some embodiments, the second expansion is performed for about 15 days.

[00215] 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. In some embodiments, the steps of the method are completed within a period of about 32 days.

[00216] In some embodiments, the second expansion is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third

culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00217] In some embodiments, the second expansion is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00218] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00219] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00220] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00221] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00222] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 1 day, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00223] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00224] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00225] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00226] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00227] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 2 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00228] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00229] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00230] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00231] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00232] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 3 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00233] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00234] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00235] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00236] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00237] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 4 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00238] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00239] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00240] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00241] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00242] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 5 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00243] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00244] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00245] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00246] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00247] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 6 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00248] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 3 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00249] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 4 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00250] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 5 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00251] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 6 days,

and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00252] In some embodiments, the step of culturing the fourth population of TILs is performed by culturing the fourth population of TILs in the second culture medium for a first period of about 7 days, at the end of the first period the culture is split into a plurality of subcultures, each of the plurality of subcultures is cultured in a third culture medium comprising IL-2 for a second period of about 7 days, and at the end of the second period the plurality of subcultures are combined to provide the expanded number of TILs.

[00253] In some embodiments, the gene-editing process can be carried out at any time during the TIL expansion method, which means that the gene-editing may be carried out on TILs before, during, or after any of the steps in the expansion method; for example, during any of steps (a)-(f) or (a)-(g) outlined in the methods above, or before or after any of steps (a)-(f) or (a)-(g) outlined in the methods above. In some embodiments, the gene-editing process can be carried out more than once at any time during the TIL expansion method. According to certain embodiments, TILs are collected during a culturing step (*e.g.*, the culturing step is "paused" for at least a portion of the TILs), and the collected TILs are subjected to a gene-editing process, and, in some cases, subsequently reintroduced back into the culturing step (*e.g.*, back into the culture medium) to continue the culturing step, so that at least a portion of the therapeutic population of TILs that are eventually transferred to the infusion bag are permanently gene-edited.

[00254] It should be noted that alternative embodiments of the expansion process may differ from the methods shown above; *e.g.*, alternative embodiments may not have the same steps (a)-(f) or (a)-(g), or may have a different number of steps. Regardless of the specific embodiment, the gene-editing process may be carried out at any time during the TIL expansion method. For example, alternative embodiments may include more than two culturing steps, and it is possible that gene-editing may be conducted on the TILs during a third or fourth culturing step, etc.

[00255] In some embodiments, gene-editing is performed while the TILs are still in the culture medium and while the culturing step is being carried out, *i.e.,* they are not necessarily "removed" from the culturing step in order to conduct gene-editing. According to some embodiments, gene-editing is performed on TILs that are collected from the culture medium, and following the gene-editing process those TILs are subsequently be placed back into the culture medium.

[00256] In some embodiments, the step of gene-editing at least a portion of the second or third population of TILs comprises performing a sterile electroporation step on the second or third population of TILs.

[00257] In some embodiments, the sterile electroporation step mediates the transfer of at least one gene editor. According to some embodiments, the gene editor is a TALE nuclease system for modulating the expression of at least one protein. According to some embodiments, the TALE nuclease system downmodulates expression of PD-1. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of CTLA-4. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of LAG-3. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of CISH. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of CBL-B. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of TIGIT. According to some embodiments, the resulting TILs are PD-1 knockout TILs. According to some embodiments, the resulting TILs are CTLA-4 knockout TILs. According to some embodiments, the resulting TILs are LAG-3 knockout TILs. According to some embodiments, the resulting TILs are CISH knockout TILs. According to some embodiments, the resulting TILs are CBL-B knockout TILs. According to some embodiments, the resulting TILs are TIGIT knockout TILs. According to some embodiments, the resulting TILs exhibit downmodulated expression of PD-1 and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CTLA-4 and downmodulated expression of one or more of PD-1, LAG-3, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of LAG-3 and downmodulated expression of one or more of PD-1, CTLA-4, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CISH and downmodulated expression of one or more of PD-1, LAG-3, CTLA-4, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CBL-B and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, TIGIT and PD-1. According to some embodiments, the resulting TILs are PD-1/CTLA-4 double knockout TILs. According to some embodiments, the resulting TILs are PD-1/LAG-3 double knockout TILs. According to some embodiments, the resulting TILs are PD-1/CISH double knockout TILs. According to some embodiments, the resulting TILs are PD-1/CBL-B double knockout TILs. According to some embodiments,

the resulting TILs are PD-1/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/LAG-3 double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/CISH double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/CISH double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CISH/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are CISH/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CBL-B/TIGIT double knockout TILs.

[00258] In some embodiments, the step of gene-editing further comprises a resting step. According to some embodiments, the resting step comprises incubating the fourth population of TILs at about 30-40 °C with about 5% CO_2. According to some embodiments, the resting step is carried out at about 30°C, about 30.5°C, about 31°C, about 31.5°C, about 32°C, about 32.5°C, about 33°C, about 33.5°C, about 34°C, about 34.5°C, about 35°C, about 35.5°C, about 36°C, about 36.5°C, about 37°C, about 37.5°C, about 38°C, about 38.5°C, about 39°C, about 39.5°C, about 40°C. According to some embodiments, the resting step is carried out 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 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about 15 hours to about 23 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 15 hours to about 23 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 15 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 16 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth

population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 17 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 18 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 19 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 20 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 21 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 22 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 23 hours at about 30°C.

[00259] 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.

[00260] In some embodiments, the tumor tissue is from a dissected tumor.

[00261] In some embodiments, the dissected tumor is less than 8 hours old.

[00262] In some embodiments, the tumor tissue is selected from the group consisting of melanoma tumor tissue, head and neck tumor tissue, breast tumor tissue, renal tumor tissue, pancreatic tumor tissue, glioblastoma tumor tissue, lung tumor tissue, colorectal tumor tissue, sarcoma tumor tissue, triple negative breast tumor tissue, cervical tumor tissue, ovarian tumor tissue, and HPV-positive tumor tissue.

[00263] In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2.5

mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 3 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 3.5 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 4 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 4.5 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 5 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 5.5 mm to 6 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2.5 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 3 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 3.5 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 4 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 4.5 mm to 5 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 4 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2 mm to 4 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2.5 mm to 4 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 3 mm to 4 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 3.5 mm to 4 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 3 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2 mm to 3 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 2.5 mm to 3 mm. In some embodiments, the tumor tissue is fragmented into approximately spherical fragments having a diameter of about 1.5 mm to 2 mm.

[00264] In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 1.5 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 2 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 2.5 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 3 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 3.5 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 4 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 4.5 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 5 mm and a longest edge length of about 6 mm. In some embodiments, the tumor tissue is fragmented into generally rectangular fragments having a shortest edge length of at least 5.5 mm and a longest edge length of about 6 mm.

[00265] In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 3 mm or about 6 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 3 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 3.5 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 4 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 4.5 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 5 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 5.5 mm. In some embodiments, the tumor tissue is fragmented into generally cubical fragments having edge lengths of about 6 mm.

[00266] In some embodiments, the present invention provides a therapeutic population of tumor infiltrating lymphocytes (TILs) product produced by a method as described herein.

[00267] In some embodiments, the present invention provides a method for treatment cancer in a patient comprising administering to the patient an effective amount of the therapeutic population of TILs produced by a method as described herein. In some embodiments, the cancer is selected from the group consisting of glioblastoma (GBM), gastrointestinal cancer, melanoma, metastatic melanoma, ovarian cancer, endometrial cancer, thyroid cancer, colorectal cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, endometrial cancer, cholangiocarcinoma, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), renal cancer, renal cell carcinoma, multiple myeloma, chronic lymphocytic leukemia, acute lymphoblastic leukemia, diffuse large B cell lymphoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma, follicular lymphoma, and mantle cell lymphoma. In some embodiments, the cancer is selected from the group consisting of cutaneous melanoma, ocular melanoma, uveal melanoma, conjunctival malignant melanoma, metastatic melanoma, pleomorphic xanthoastrocytoma, dysembryoplastic neuroepithelial tumor, ganglioglioma, and pilocytic astrocytoma, endometrioid adenocarcinoma with significant mucinous differentiation (ECMD), papillary thyroid carcinoma, serous low-grade or borderline ovarian carcinoma, hairy cell leukemia, and Langerhans cell histiocytosis.

[00268] 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 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 1000 IU/mL and 5000 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 5000 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 5000 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 5000 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 5000 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 5000 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 5000 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 5000 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 1000 IU/mL and 4000 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 4000 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 4000 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 4000 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 4000 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 4000 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 1000 IU/mL and 3000 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 3000 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 3000 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 3000 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 1000 IU/mL and 2000 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 2000 IU/mL in the cell culture medium in the first expansion.

[00269] 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.

[00270] In some embodiments, the first cell culture medium and/or the second cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist.

[00271] 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.

[00272] 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.

[00273] In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the TILs or PBL product to the patient. In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after the administration of the TILs or PBL product to the patient. In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the same day as administration of the TILs or PBL product to the patient. In some embodiments, the IL-2 regimen comprises aldesleukin, nemvaleukin, or a biosimilar or variant thereof.

[00274] In some embodiments, the therapeutically effective amount of TILs product comprises from about 2.3×10^{10} to about 13.7×10^{10} TILs.

[00275] In some embodiments, the second population of TILs is at least 50-fold greater in number than the first population of TILs.

C. PD-1

[00276] One of the most studied targets for the induction of checkpoint blockade is the programmed death receptor (PD1 or PD-1, also known as PDCD1), a member of the CD28 super family of T-cell regulators. Its ligands, PD-L1 and PD-L2, are expressed on a variety of tumor cells, including melanoma. The interaction of PD-1 with PD-L1 inhibits T-cell effector function, results in T-cell exhaustion in the setting of chronic stimulation, and induces T-cell apoptosis in the tumor microenvironment. PD-1 may also play a role in tumor-specific escape from immune surveillance.

[00277] According to particular embodiments, expression of PD-1 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein, wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of PD-1. As described in more detail below, the gene-editing process may involve the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as PD-1. For example, a TALEN method may be used to silence or reduce the expression of PD-1 in the TILs.

D. CTLA-4

[00278] CTLA-4 expression is induced upon T-cell activation on activated T-cells, and competes for binding with the antigen presenting cell activating antigens CD80 and CD86. Interaction of CTLA-4 with CD80 or CD86 causes T-cell inhibition and serves to maintain balance of the immune response. However, inhibition of the CTLA-4 interaction with CD80 or CD86 may prolong T-cell activation and thus increase the level of immune response to a cancer antigen.

[00279] According to particular embodiments, expression of CTLA-4 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. According to particular embodiments, expression of both PD-1 and CTLA-4 in TILs are silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of CTLA-4. As described in more detail below, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as CTLA-4. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of CTLA-4 in the TILs. In some embodiments, a TALEN method may be used to silence or reduce the expression of PD-1 and CTLA-4 in the TILs.

E. LAG-3

[00280] Lymphocyte activation gene-3 (LAG-3, CD223) is expressed by T cells and natural killer (NK) cells after major histocompatibility complex (MHC) class II ligation. Although its mechanism remains unclear, its modulation causes a negative regulatory effect over T cell function, preventing tissue damage and autoimmunity. Thus, LAG-3 blockade may improve anti-tumor responses. *See, e.g.,* Marin-Acevedo *et al., Journal of Hematology & Oncology* (2018) 11:39.

[00281] According to particular embodiments, expression of LAG-3 in TILs is silenced or reduced in accordance with compositions and methods of the present invention. According to particular embodiments, expression of both PD-1 and LAG-3 in TILs are silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of LAG-3. As described in more detail below, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as LAG-3. According to particular embodiments, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of LAG-3 in the TILs. In some embodiments, a TALEN method may be used to silence or reduce the expression of PD-1 and LAG-3 in the TILs.

F. Cish

[00282] Cish, a member of the suppressor of cytokine signaling (SOCS) family, is induced by TCR stimulation in CD8+ T cells and inhibits their functional avidity against tumors. Genetic deletion of Cish in CD8+ T cells may enhance their expansion, functional avidity, and cytokine polyfunctionality, resulting in pronounced and durable regression of established tumors. *See, e.g.,* Palmer *et al., Journal of Experimental Medicine*, 212 (12): 2095 (2015).

[00283] According to particular embodiments, expression of Cish in TILs is silenced or reduced in accordance with compositions and methods of the present invention. According to particular embodiments, expression of both PD-1 and Cish in TILs are silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing

or repressing the expression of Cish. As described in more detail below, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as Cish. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of Cish in the TILs. In some embodiments, a TALEN method may be used to silence or reduce the expression of PD-1 and Cish in the TILs.

G. CBL-B

[00284] CBLB (or CBL-B) is a E3 ubiquitin-protein ligase and is a negative regulator of T cell activation. Bachmaier, *et al., Nature*, 2000, 403, 211–216; Wallner, et al., *Clin. Dev. Immunol.* **2012,** 692639.

[00285] According to particular embodiments, expression of CBL-B in TILs is silenced or reduced in accordance with compositions and methods of the present invention. According to particular embodiments, expression of both PD-1 and CBL-B in TILs are silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of CBL-B. As described in more detail below, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as CBL-B. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of PKA in the TILs. In some embodiments, CBL-B is silenced using a TALEN knockout. In some embodiments, CBL-B is silenced using a TALE-KRAB transcriptional inhibitor knock in. More details on these methods can be found in Boettcher and McManus, *Mol. Cell Review*, **2015,** *58,* 575-585. In some embodiments, a TALEN method may be used to silence or reduce the expression of PD-1 and CBL-B in the TILs.

H. TIGIT

[00286] TIGIT is a cell surface protein that is expressed on regulatory, memory and activated T cells. TIGIT belongs to the poliovirus receptor (PVR) family of immunoglobulin proteins and suppresses T-cell activation. (Yu *et al., Nat Immunol.*, 2009, 10(1):48-57).

According to particular embodiments, expression of TIGIT in TILs is silenced or reduced in accordance with compositions and methods of the present invention. According to particular embodiments, expression of both PD-1 and TIGIT in TILs are silenced or reduced in accordance with compositions and methods of the present invention. For example, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs may be carried out in accordance with any embodiment of the methods described herein (e.g., process 2A or the methods shown in Figures 20 and 21), wherein the method comprises gene-editing at least a portion of the TILs by silencing or repressing the expression of TIGIT. As described in more detail below, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at an immune checkpoint gene, such as TIGIT. For example, a CRISPR method, a TALE method, or a zinc finger method may be used to silence or repress the expression of PKA in the TILs. In some embodiments, TIGIT is silenced using a TALEN knockout. In some embodiments, TIGIT is silenced using a TALE-KRAB transcriptional inhibitor knock in. More details on these methods can be found in Boettcher and McManus, *Mol. Cell Review*, **2015,** *58,* 575-585. In some embodiments, a TALEN method may be used to silence or reduce the expression of PD-1 and TIGIT in the TILs.

III. Gene-Editing Methods

[00287] As discussed above, embodiments of the present invention provide tumor infiltrating lymphocytes (TILs) that have been genetically modified via gene-editing to enhance their therapeutic effect. Embodiments of the present invention embrace genetic editing through nucleotide insertion (RNA or DNA) into a population of TILs for inhibition of the expression of one or more proteins. Embodiments of the present invention also provide methods for expanding TILs into a therapeutic population, wherein the methods comprise gene-editing the TILs. There are several gene-editing technologies that may be used to genetically modify a population of TILs, which are suitable for use in accordance with the present invention.

[00288] In some embodiments, a method of genetically modifying a population of TILs includes the step of stable incorporation of genes for production of one or more proteins. In an embodiment, a method of genetically modifying a population of TILs includes the step of retroviral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of lentiviral transduction. Lentiviral transduction systems are known in the art and are described, *e.g.*, in Levine, *et al., Proc. Nat'l Acad. Sci.* **2006,** *103,* 17372-77; Zufferey, *et al., Nat. Biotechnol.* **1997,** *15,* 871-75; Dull, *et*

al., *J. Virology* **1998,** *72*, 8463-71, and U.S. Patent No. 6,627,442, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of gamma-retroviral transduction. Gamma-retroviral transduction systems are known in the art and are described, *e.g.*, Cepko and Pear, *Cur. Prot. Mol. Biol.* **1996,** 9.9.1-9.9.16, the disclosure of which is incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of transposon-mediated gene transfer. Transposon-mediated gene transfer systems are known in the art and include systems wherein the transposase is provided as DNA expression vector or as an expressible RNA or a protein such that long-term expression of the transposase does not occur in the transgenic cells, for example, a transposase provided as an mRNA (*e.g.*, an mRNA comprising a cap and poly-A tail). Suitable transposon-mediated gene transfer systems, including the salmonid-type Tel-like transposase (SB or Sleeping Beauty transposase), such as SB10, SB11, and SB100x, and engineered enzymes with increased enzymatic activity, are described in, *e.g.*, Hackett, *et al.*, *Mol. Therapy* **2010,** *18,* 674-83 and U.S. Patent No. 6,489,458, the disclosures of each of which are incorporated by reference herein.

[00289] In an embodiment, a method of genetically modifying a population of TILs includes the step of stable incorporation of genes for production or inhibition (*e.g.*, silencing) of one or more proteins. In an embodiment, a method of genetically modifying a population of TILs includes the step of electroporation. Electroporation methods are known in the art and are described, *e.g.*, in Tsong, *Biophys. J.* **1991,** *60,* 297-306, and U.S. Patent Application Publication No. 2014/0227237 A1, the disclosures of each of which are incorporated by reference herein. Other electroporation methods known in the art, such as those described in U.S. Patent Nos. 5,019,034; 5,128,257; 5,137,817; 5,173,158; 5,232,856; 5,273,525; 5,304,120; 5,318,514; 6,010,613 and 6,078,490, the disclosures of which are incorporated by reference herein, may be used. In an embodiment, the electroporation method is a sterile electroporation method. In an embodiment, the electroporation method is a pulsed electroporation method. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein the sequence of at least three DC electrical pulses has one, two, or three of the following characteristics: (1) at least two of the at least three pulses differ from each other in pulse amplitude; (2) at least two of the at least three pulses

differ from each other in pulse width; and (3) a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein at least two of the at least three pulses differ from each other in pulse amplitude. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein at least two of the at least three pulses differ from each other in pulse width. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to alter, manipulate, or cause defined and controlled, permanent or temporary changes in the TILs, comprising the step of applying a sequence of at least three single, operator-controlled, independently programmed, DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to the TILs, wherein a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses. In an embodiment, the electroporation method is a pulsed electroporation method comprising the steps of treating TILs with pulsed electrical fields to induce pore formation in the TILs, comprising the step of applying a sequence of at least three DC electrical pulses, having field strengths equal to or greater than 100 V/cm, to TILs, wherein the sequence of at least three DC electrical pulses has one, two, or three of the following characteristics: (1) at least two of the at least three pulses differ from each other in pulse amplitude; (2) at least two of the at least three pulses differ from each other in pulse width; and (3) a first pulse interval for a first set of two of the at least three pulses is different from a second pulse interval for a second set of two of the at least three pulses, such that induced pores are sustained for a relatively long period of time, and such that viability of the TILs is maintained. In an embodiment, a method of genetically modifying a population of TILs includes the step of calcium phosphate transfection. Calcium phosphate transfection methods (calcium phosphate DNA precipitation, cell surface coating, and endocytosis) are known in the art and are described in Graham and van der Eb, *Virology* **1973,** *52,* 456-467; Wigler, *et al., Proc. Natl. Acad. Sci.* **1979,** *76,* 1373-1376; and

Chen and Okayarea, *Mol. Cell. Biol.* **1987,** *7,* 2745-2752; and in U.S. Patent No. 5,593,875, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of liposomal transfection. Liposomal transfection methods, such as methods that employ a 1:1 (w/w) liposome formulation of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE) in filtered water, are known in the art and are described in Rose, *et al.*, *Biotechniques* **1991,** *10,* 520-525 and Felgner, *et al.*, *Proc. Natl. Acad. Sci. USA*, **1987,** *84,* 7413-7417 and in U.S. Patent Nos. 5,279,833; 5,908,635; 6,056,938; 6,110,490; 6,534,484; and 7,687,070, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of transfection using methods described in U.S. Patent Nos. 5,766,902; 6,025,337; 6,410,517; 6,475,994; and 7,189,705; the disclosures of each of which are incorporated by reference herein.

[00290] According to an embodiment, the gene-editing process may comprise the use of a programmable nuclease that mediates the generation of a double-strand or single-strand break at one or more immune checkpoint genes. Such programmable nucleases enable precise genome editing by introducing breaks at specific genomic loci, *i.e.,* they rely on the recognition of a specific DNA sequence within the genome to target a nuclease domain to this location and mediate the generation of a double-strand break at the target sequence. A double-strand break in the DNA subsequently recruits endogenous repair machinery to the break site to mediate genome editing by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). Thus, the repair of the break can result in the introduction of insertion/deletion mutations that disrupt (*e.g.*, silence, repress, or enhance) the target gene product.

[00291] Major classes of nucleases that have been developed to enable site-specific genomic editing include zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and CRISPR-associated nucleases (*e.g.*, CRISPR/Cas9). These nuclease systems can be broadly classified into two categories based on their mode of DNA recognition: ZFNs and TALENs achieve specific DNA binding via protein-DNA interactions, whereas CRISPR systems, such as Cas9, are targeted to specific DNA sequences by a short RNA guide molecule that base-pairs directly with the target DNA and by protein-DNA interactions. *See, e.g.,* Cox *et al.*, *Nature Medicine,* 2015, Vol. 21, No. 2.

[00292] Non-limiting examples of gene-editing methods that may be used in accordance with TIL expansion methods of the present invention include CRISPR methods, TALE methods, and ZFN methods,

which are described in more detail below. According to an embodiment, a method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in WO 2018/081473 A1, WO 2018/129332 A1, or WO 2018/182817 A1, wherein the method further comprises gene-editing at least a portion of the TILs by one or more of a CRISPR method, a TALE method or a ZFN method, in order to generate TILs that can provide an enhanced therapeutic effect. According to an embodiment, gene-edited TILs can be evaluated for an improved therapeutic effect by comparing them to non-modified TILs *in vitro, e.g.,* by evaluating *in vitro* effector function, cytokine profiles, etc. compared to unmodified TILs.

[00293] In some embodiments of the present invention, electroporation is used for delivery of a gene-editing system, such as CRISPR, TALEN, and ZFN systems. In some embodiments of the present invention, the electroporation system is a flow electroporation system. An example of a suitable flow electroporation system suitable for use with some embodiments of the present invention is the commercially-available MaxCyte STX system. There are several alternative commercially-available electroporation instruments which may be suitable for use with the present invention, such as the AgilePulse system or ECM 830 available from BTX-Harvard Apparatus, Cellaxess Elektra (Cellectricon), Nucleofector (Lonza/Amaxa), GenePulser MXcell (BIORAD), iPorator-96 (Primax) or siPORTer96 (Ambion). In some embodiments of the present invention, the electroporation system forms a closed, sterile system with the remainder of the TIL expansion method. In some embodiments of the present invention, the electroporation system is a pulsed electroporation system as described herein, and forms a closed, sterile system with the remainder of the TIL expansion method.

1. TALE Methods

[00294] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein or as described in WO 2018/081473 A1, WO 2018/129332 A1, or WO 2018/182817 A1, wherein the method further comprises gene-editing at least a portion of the TILs by a TALE method. According to particular embodiments, the use of a TALE method during the TIL expansion process causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a TALE method during the TIL expansion process causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

[00295] TALE stands for "Transcription Activator-Like Effector" proteins, which include TALENs ("Transcription Activator-Like Effector Nucleases"). A method of using a TALE system for gene-editing may also be referred to herein as a TALE method. TALEs are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair. TALE specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues (RVDs). Modular TALE repeats are linked together to recognize contiguous DNA sequences. A specific RVD in the DNA-binding domain recognizes a base in the target locus, providing a structural feature to assemble predictable DNA-binding domains. The DNA binding domains of a TALE are fused to the catalytic domain of a type IIS FokI endonuclease to make a targetable TALE nuclease. To induce site-specific mutation, two individual TALEN arms, separated by a 14-20 base pair spacer region, bring FokI monomers in close proximity to dimerize and produce a targeted double-strand break.

[00296] Several large, systematic studies utilizing various assembly methods have indicated that TALE repeats can be combined to recognize virtually any user-defined sequence. Custom-designed TALE arrays are also commercially available through Cellectis Bioresearch (Paris, France), Transposagen Biopharmaceuticals (Lexington, KY, USA), and Life Technologies (Grand Island, NY, USA). TALE and TALEN methods suitable for use in the present invention are described in U.S. Patent Application Publication Nos. US 2011/0201118 A1; US 2013/0117869 A1; US 2013/0315884 A1; US 2015/0203871 A1 and US 2016/0120906 A1, the disclosures of which are incorporated by reference herein.

[00297] Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a TALE method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, PKA, CBL-B, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3.

[00298] Non-limiting examples of TALE-nucleases targeting the PD-1 gene are provided in the following table. In these examples, the targeted genomic sequences contain two 17-base pair (bp) long sequences (referred to as half targets, shown in upper case letters) separated by a 15-bp spacer (shown in lower case letters). Each half target is recognized by repeats of half TALE-nucleases listed in the table. Thus, according to particular embodiments, TALE-nucleases according to the invention recognize and cleave the target sequence selected from the group consisting of: SEQ ID NO: 127 and SEQ ID NO: 128.

TALEN sequences and gene-editing methods are also described in Gautron *et al., Molecular Therapy: Nucleic Acids* Dec. 2017, Vol. 9:312-321, which is incorporated by reference herein.

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

[00299] Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a TALE method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL12, IL-15, and IL-21.

[00300] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a TALE method, and which may be used in accordance with embodiments of the present invention, are described in U.S. Patent No. 8,586,526, which is incorporated by reference herein.

2. Cas-CLOVER Methods

[00301] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a Cas-CLOVER method. According to particular embodiments, the use of a Cas-CLOVER method during the TIL expansion process causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs. Alternatively, the use of a Cas-CLOVER method during the TIL expansion process causes expression of one or more immune checkpoint genes to be enhanced in at least a portion of the therapeutic population of TILs.

[00302] Cas-CLOVER is a dimeric, high-fidelity site-specific nuclease (SSN) that consists of a fusion of catalytically dead SpCas9 (dCas9) with the nuclease domain from a Clostridium Clo051 type IIs restriction endonuclease (Madison, et al., "Cas-CLOVER is a novel high-fidelity nuclease for safe and robust generation of T SCM-enriched allogeneic CAR-T cells," Molecular Therapy - Nucleic Acids, 2022). This yields a nuclease whose activity is predicated upon the dimerization of the Clo051 nuclease domain, enabled by RNA-guided recognition of two adjacent 20-nt target sequences. Unlike a paired nickase approach, e.g., when using the Cas9-D10A mutant, monomeric Cas-CLOVER does not introduce a nick or a DSB. Cas-CLOVER has been shown to have low off-target nuclease activity.

[00303] Exemplary Cas-CLOVER systems include those described in WO2019/126578, the contents of which are incorporated herein by reference in their entirety. In embodiments, the Cas-CLOVER system comprises a fusion protein comprising, consisting essentially of, or consisting of a DNA localization component and an effector molecule.

[00304] *DNA localization components*

[00305] In embodiments, the DNA localization components are capable of binding a specific DNA sequence. In embodiments, the DNA localization component is selected from, for example, a DNA-binding oligonucleotide, a DNA-binding protein, a DNA binding protein complex, and combinations thereof. Other suitable DNA binding components will be recognized by one of ordinary skill in the art.

[00306] In embodiments, the DNA localization components comprise an oligonucleotide directed to a specific locus or loci in the genome. The oligonucleotide may be selected from DNA, RNA, DNA/RNA hybrids, and combinations thereof.

[00307] In embodiments, the DNA localization components comprise a nucleotide binding protein or protein complex that binds an oligonucleotide when bound to a target DNA. The protein or protein complex may be capable of recognizing a feature selected from RNA-DNA heteroduplexes, R-loops, or combinations thereof. In embodiments, the DNA localization component comprises a protein or protein complex capable of recognizing an R-loop selected from Cas9, Cascade complex, RecA, RNase H, RNA polymerase, DNA polymerase, or a combination thereof. In embodiments, the DNA localization component comprises an engineered protein capable of binding to target DNA. In embodiments, the DNA localization component comprises a protein capable of binding a DNA sequence selected from meganuclease, zinc finger array, transcription activator-like (TAL) array, and combinations thereof. In embodiments, the DNA localization component comprises a protein that contains a naturally occurring

DNA binding domain. In embodiments, the DNA localization component comprises a bZIP domain, a Helix-loop-helix, a Helix-turn-helix, a HMG-box, a Leucine zipper, a Zinc finger, or a combination thereof. In embodiments, the DNA localization component comprises an oligonucleotide directed to a specific locus in the genome. Exemplary oligonucleotides include, but are not limited to, DNA, RNA, DNA/RNA hybrids, and any combination thereof. In embodiments, the DNA localization component comprises a protein or a protein complex capable of recognizing a feature selected from RNA-DNA heteroduplexes, R-loops, and any combination thereof. Exemplary proteins or protein complexes capable of recognizing an R-loop include, but are not limited to, Cas9, Cascade complex, RecA, RNase H, RNA polymerase, DNA polymerase, and any combination thereof. In embodiments, the protein or protein complex capable of recognizing an R-loop comprises Cas9. In embodiments, the DNA localization component comprises a protein capable of binding a DNA sequence selected from meganuclease, Zinc Finger array, TAL array, and any combination thereof. In embodiments, the DNA localization component comprises an oligonucleotide directed to a target location in a genome and a protein capable of binding to a target DNA sequence.

[00308] In embodiments, the DNA localization components comprise, consist essentially of, or consist of, at least one guide RNA (gRNA). In embodiments, the DNA localization components comprise, consist essentially of, or consist of, two gRNAs, wherein a first gRNA specifically binds to a first strand of a double-stranded DNA target sequence and a second gRNA specifically binds to a second strand of the double-stranded DNA target sequence. Alternatively, in embodiments, DNA localization components comprise, consist essentially of, or consist of, a DNA binding domain of a transcription activator-like effector nuclease (TALEN, also referred to as a TAL protein). In embodiments DNA localization components comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from Xanthomonas or Ralstonia.

[00309] *Effector molecules*

[00310] In embodiments, effector molecules are capable of a predetermined effect at a specific locus in the genome. Exemplary effector molecules, but are not limited to, a transcription factor (activator or repressor), chromatin remodeling factor, nuclease, exonuclease, endonuclease, transposase, methytransferase, demethylase, acetyltransferase, deacetylase, kinase, phosphatase, integrase, recombinase, ligase, topoisomerase, gyrase, helicase, fluorophore, or any combination thereof.

[00311] In embodiments, effector molecules comprise a transposase. In embodiments, effector molecules comprise a PB transposase (PBase). In embodiments, effector molecules comprise a nuclease. Non-limiting examples of nucleases include restriction endonucleases, homing endonucleases, S1 nuclease, mung bean nuclease, pancreatic DNase I, micrococcal nuclease, yeast HO endonuclease, or any combination thereof. In certain embodiments, the effector molecule comprises a restriction endonuclease. In certain embodiments, the effector molecule comprises a Type IIS restriction endonuclease. In embodiments, effector molecules comprise an endonuclease. Non-limiting examples of the endonuclease include AciI, Mn1I, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, Mbo1I, My1I, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I and Clo051. In embodiments, the effector molecule comprises BmrI, BfiI, or Clo051.

[00312] In embodiments, effector molecules comprise, consist essentially of, or consist of, a homodimer or a heterodimer. In embodiments, effector molecules comprise, consist essentially of, or consist of, a nuclease, optionally an endonuclease. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a Cas9, a Cas9 nuclease domain or a fragment thereof. In embodiments, the Cas9 is a catalytically inactive or "inactivated" Cas9 (dCas9 (SEQ ID NO: 302 and 303 of WO2019/126578)). In embodiments, the Cas9 is a catalytically inactive or "inactivated" nuclease domain of Cas9. In embodiments, the dCas9 is encoded by a shorter sequence that is derived from a full length, catalytically inactivated, Cas9, referred to herein as a "small" dCas9 or dSaCas9 (SEQ ID NO: 23 of WO2019/126578).

[00313] In embodiments of the fusion protein, the effector molecule comprises, consists essentially of, or consists of a homodimer or a heterodimer of one or more Type II nucleases. In embodiments of the fusion protein, the effector molecule comprises, consists essentially of, or consists of a homodimer or a heterodimer of a Type II nuclease. In embodiments, the Type II nuclease comprises one or more of AciI, Mn1I, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, Mbo1I, My1I, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I or Clo051.

[00314] In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, Clo051, BfiI or BmrI. In

embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a Cas9, a Cas9 nuclease domain or a fragment thereof that forms a heterodimer with Clo051, Bfil or Bmrl. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a catalytically-inactive form of Cas9 (e.g. dCas9 or dSaCas9) or a fragment thereof that forms a heterodimer with Clo051. An exemplary Clo05 l nuclease domain may comprise, consist essentially of or consist of, the amino acid sequence of:
EGIKSNISLLKDELRGQISHISHEYLSLIDLAFDSKQNRLFEMKVLELLVNEYGFKGRH
LGGSRKPDGIVYSTTLEDNFGIIVDTKAYSEGYSLPISQADEMERYVRENSNRDEEVN
PNKWWENFSEEVKKYYFVFISGSFKGKFEEQLRRLSMTTGVNGSAVNVVNLLLGAE
KIRSGEMTIEELERAMFNNSEFILKY (SEQ ID NO:238).

[00315] In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from Xanthomonas or Ralstonia. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from Xanthomonas or Ralstonia that forms a homodimer or a heterodimer with Clo051, Bfil or Bmrl. In embodiments, effector molecules, including those effector molecules comprising a homodimer or a heterodimer, comprise, consist essentially of, or consist of, a DNA-binding domain of a TALEN, or TAL protein, derived from Xanthomonas or Ralstonia that forms a homodimer or a heterodimer with Clo051.

[00316] *Linkages*

[00317] In embodiments, the fusion protein comprises, consists essentially of, or consists of, a DNA localization component and an effector molecule. In embodiments, the nucleic acid sequences encoding one or more components of the fusion protein can be operably linked, for example, in an expression vector. In embodiments, the fusion proteins are chimeric proteins. In embodiments, the fusion proteins are encoded by one or more recombinant nucleic acid sequences. In embodiments, the fusion proteins also include a linker region to operatively-link two components of the fusion protein. For example, in embodiments, the fusion protein comprises, consists essentially of, or consists, of a DNA localization component and an effector molecule, operatively-linked by a linker region. In embodiments, the DNA localization component, the linker region, and the effector molecule can be encoded by one or more nucleic acid sequences inserted into an expression cassette and/or expression vector such that

translation of the nucleic acid sequence results in the fusion protein. in embodiments, the fusion protein can comprise a non-covalent linkage between the DNA localization component and the effector molecule. The non-covalent linkage can comprise an antibody, an antibody fragment, an antibody mimetic, or a scaffold protein.

[00318] *Fusion proteins*

[00319] In embodiments, the DNA localization component comprises, consists essentially of or consists of, at least one gRNA, and the effector molecule comprises, consists essentially of, or consists of a Cas9, a Cas9 nuclease domain, or a fragment thereof. In embodiments, the DNA localization component comprises, consists essentially of, or consists of, at least one gRNA, and the effector molecule comprises, consists essentially of, or consist of an inactivated Cas9 (dCas9) or an inactivated nuclease domain. In embodiments, the DNA localization component comprises, consists essentially of, or consists of, at least one gRNA, and the effector molecule comprises, consists essentially of, or consist of an inactivated small Cas9 (dSaCas9). In embodiments, the effector molecule comprises, consists essentially of, or consists of a Cas9, dCas9, dSaCas9, or nuclease domain thereof, and a second endonuclease. The second endonuclease can comprise, consist essentially of, or consist of a Type IIS endonuclease, including, but not limited to, one or more of AciI, Mn1I, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, Mbo1I, My1I, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I, FokI or Clo051.

[00320] In embodiments of the fusion proteins, the DNA localization component comprises, consists essentially of, or consists of, a DNA-binding domain of a transcription activator-like effector nuclease (TALEN, also referred to as a TAL protein), and the effector molecule comprises, consists essentially of, or consists of, an endonuclease. In embodiments of the fusion proteins of the disclosure, the DNA localization component comprises, consists essentially of, or consists of, a DNA-binding domain of a TALEN, or TAL protein, derived from Xanthomonas or Ralstonia, and the effector molecule comprises, consists essentially of, or consists of, a Type IIS endonuclease, including, but not limited to, one or more of AciI, Mn1I, AlwI, BbvI, BccI, BceAI, BsmAI, BsmFI, BspCNI, BsrI, BtsCI, HgaI, HphI, HpyAV, Mbo1I, My1I, PleI, SfaNI, AcuI, BciVI, BfuAI, BmgBI, BmrI, BpmI, BpuEI, BsaI, BseRI, BsgI, BsmI, BspMI, BsrBI, BsrBI, BsrDI, BtgZI, BtsI, EarI, EciI, MmeI, NmeAIII, BbvCI, Bpu10I, BspQI, SapI, BaeI, BsaXI, CspCI, BfiI, MboII, Acc36I or Clo051.

[00321] In certain embodiments, an exemplary dCas9-Clo051 fusion protein may comprise, consist essentially of or consist of the amino acid sequence of SEQ ID NO: 305 or 307 of WO2019/126578 or the nucleic acid sequence of SEQ ID NO: 306 or 308 of WO2019/126578.

[00322] *Constructs*

[00323] In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a dCas9 and Clo051. In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a dSaCas9 and Clo051. In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a Xanthomonas-TALE and Clo051. In embodiments, the nuclease domain comprises, consists essentially of, or consists of, a Ralstonia-TALE and Clo051. In embodiments, the fusion protein comprises dCas9-Clo051, dSaCas9-Clo051, Xanthomonas-TALE-Clo051, or Ralstonia-TALE-Clo051. In embodiments, a vector encoding the fusion protein comprises Csy4-T2A-Clo051-G4Slinker-dCas9 (Streptoccocus pyogenes) or pRT1-Clo051-dCas9 double NLS.

[00324] According to some embodiments, a Cas-CLOVER system comprises a fusion protein comprising a DNA localization component and an effector molecule, wherein the DNA localization component hybridizes to a target sequence of a DNA molecule in a TIL, wherein the DNA molecule encodes and the TIL expresses at least one immune checkpoint molecule, and the effector molecule cleaves the DNA molecule, whereby expression of the at least one immune checkpoint molecule is altered.

[00325] According to particular embodiments, a Cas-CLOVER method comprises silencing or reducing the expression of one or more immune checkpoint genes in TILs by introducing a Cas-CLOVER system (*e.g.*, dCas9-Clo051, dSaCas9-Clo051, Xanthomonas-TALE-Clo051, or Ralstonia-TALE-Clo051 fusion protein) specific to a target DNA sequence of the immune checkpoint gene(s). The fusion protein may be delivered as DNA, mRNA, protein. Upon contact of the genome with the Cas-CLOVER system, one or more strand of the target double-stranded DNA may be cut. If the cut is made in the presence of one or more DNA repair pathways or components thereof, the Cas-CLOVER method either interrupts gene expression or modifies the genomic sequence by insertion, deletion, or substitution of one or more base pairs. DSBs may be repaired in the cells by non-homologous end joining (NHEJ), a mechanism which frequently causes insertions or deletions (indels) in the DNA. Indels often lead to frameshifts, creating loss of function alleles; for example, by causing premature stop codons within the open reading frame (ORF) of the targeted gene. According to certain embodiments, the result is a loss-of-function mutation within the targeted immune checkpoint gene.

[00326] Alternatively, DSBs induced by Cas-CLOVER systems may be repaired by homology-directed repair (HDR) instead of NHEJ. While NHEJ-mediated DSB repair often disrupts the open reading frame of the gene, homology directed repair (HDR) can be used to generate specific nucleotide changes ranging from a single nucleotide change to large insertions. According to some embodiments, HDR is used for gene editing immune checkpoint genes by delivering a DNA repair template containing the desired sequence into the TILs with the Cas-CLOVER system. The repair template preferably contains the desired edit as well as additional homologous sequence immediately upstream and downstream of the target gene (often referred to as left and right homology arms).

[00327] Non-limiting examples of genes that may be silenced or inhibited by permanently gene-editing TILs via a Cas-CLOVER method include PD-1, CTLA-4, LAG-3, HAVCR2 (TIM-3), Cish, TGFβ, 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.

[00328] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a Cas-CLOVER method, and which may be used in accordance with embodiments of the present invention, are described in WO2019126578, US2017/0107541, US2017/0114149, US2018/0187185, and U.S. Patent No. 10,415,024, the contents of which are incorporated herein by reference in their entirety. Resources for carrying out Cas-CLOVER methods, such as CLOVER mRNA and Cas-CLOVER mRNA constructs, are commercially available from companies such as Demeetra and Hera Biolabs.

[00329] According to some embodiments, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into multiple tumor fragments;

(b) adding the tumor fragments into a closed system;

(c) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 3 to 11 days

to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area;

(d) stimulating the second population of TILs by adding OKT-3 and culturing for about 1 to 3 days, wherein the transition from step (c) to step (d) occurs without opening the system;

(e) sterile electroporating the second population of TILs to effect transfer of at least one gene editor into a plurality of cells in the second population of TILs;

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

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

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

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

(j) optionally cryopreserving the harvested TIL population using a cryopreservation medium,

wherein the electroporation step comprises the delivery of at least one gene editor system comprising a Cas-CLOVER system, which at least one gene editor system modulates expression of at least one checkpoint protein in the plurality of cells of the second population of TILs.

[00330] According to some embodiments, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into multiple tumor fragments;

(b) adding the tumor fragments into a closed system;

(c) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 3 to 11 days to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area;

(d) stimulating the second population of TILs by adding OKT-3 and culturing for about 1 to 3 days to obtain the second population of TILs, wherein the transition from step (c) to step (d) occurs without opening the system;

(e) sterile electroporating the second population of TILs to effect transfer of at least one gene editor into a plurality of cells in the second population of TILs;

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

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

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

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

(j) optionally cryopreserving the harvested TIL population using a cryopreservation medium,

wherein the electroporation step comprises the delivery of at least one gene editor system comprising a Cas-CLOVER system, which at least one gene editor system inhibits expression of at least one checkpoint protein in the plurality of cells of the second population of TILs.

IV. Gene Expression Methods

[00331] In some embodiments, a method of genetically modifying a population of TILs to modulate protein expression can optionally include the step of stable incorporation of genes for production of one or more proteins. In an embodiment, a method of genetically modifying a population of TILs includes the step of viral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of retroviral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of gamma-retroviral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of adenoviral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of adeno-associated viral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of herpes simplex viral transduction. In an embodiment, a method of genetically modifying a population of TILs includes the step of poxvirus viral transduction. In some embodiments, a method of genetically modifying a population of TILs includes the step of lentiviral transduction, including lentiviral transduction using human immunodeficiency virus (HIV), including HIV-1. Lentiviral transduction systems and other suitable viral transduction systems are known in the art and are described, *e.g.*, in Levine, *et al.*, *Proc. Nat'l Acad. Sci.* **2006,** *103,* 17372-77; Zufferey, *et al., Nat. Biotechnol.* **1997,** *15,* 871-75; Dull, *et al.*, *J. Virology* **1998,** *72*, 8463-71, and U.S. Patent Nos. 5,350,674; 5,585,362; and 6,627,442, the disclosures of each of which are incorporated by reference herein. In an embodiment, a method of genetically modifying a population of TILs includes the step of gamma-retroviral transduction. Gamma-retroviral transduction systems are known in the art and are described, *e.g.*, Cepko and Pear, *Cur. Prot. Mol. Biol.* **1996,** 9.9.1-9.9.16, Hawley, *et al.*, *Gene Ther.* **1994,** *1,* 136-38; the disclosure of which is incorporated by reference herein.

1. piggyBac Methods

[00332] A method for expanding TILs into a therapeutic population may be carried out in accordance with any embodiment of the methods described herein (*e.g.*, process 2A) or as described in PCT/US2017/058610, PCT/US2018/012605, or PCT/US2018/012633, wherein the method further comprises gene-editing at least a portion of the TILs by a piggyBac method (*e.g.,* piggyBac transposons and transposases or piggyBac-like transposons and transposases). According to particular embodiments, the use of a piggyBac method during the TIL expansion process causes expression of at least one immunomodulatory composition at the cell surface of at least a portion of the therapeutic population of TILs. Alternatively, the use of a piggyBac method during the TIL expansion process causes

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

[00333] The piggyBac transposon is a mobile genetic element that efficiently transposes between the donor vector and host chromosomes. This system has almost no cargo limit, and is fully reversible, leaving no footprint in the genome after excision. The piggyBac transposon/transposase system consists of a transposase that recognizes piggyBac-specific inverted terminal repeat sequences (ITRs) located on both sides of the transposon cassette. The transposase excises the transposable element to integrate it into TT/AA chromosomal sites that are preferentially located in euchromatic regions of mammalian genomes (Ding et al. 2005; Cadinaños and Bradley 2007; Wilson et al. 2007; Wang et al. 2008; Li et al. 2011).

[00334] Exemplary piggyBac systems include those described in WO2019/046815, the contents of which are incorporated herein by reference in their entirety. In embodiments, the piggyBac system comprises a transposon/transposase system.

[00335] In embodiments, a piggyBac method comprises delivering to the TILs, (a) a nucleic acid or amino acid sequence comprising a sequence encoding a transposase enzyme and (b) a recombinant and non-naturally occurring DNA sequence comprising a DNA sequence encoding a transposon.

[00336] In embodiments, the sequence encoding a transposase enzyme is an mRNA sequence. In embodiments, the sequence encoding a transposase enzyme is a DNA sequence. In embodiments, the DNA sequence is a cDNA sequence. In embodiments, the sequence encoding a transposase enzyme is an amino acid sequence. A protein Super piggybac transposase (SPB) may be delivered following pre-incubation with transposon DNA.

[00337] *Transposons/Transposases*

[00338] Exemplary transposon/transposase systems include, but are not limited to, piggyBac transposons and transposases, Sleeping Beauty transposons and transposases, Helraiser transposons and transposases and Tol2 transposons and transposases.

[00339] The piggyBac transposase recognizes transposon-specific inverted terminal repeat sequences (ITRs) on the ends of the transposon, and moves the contents between the ITRs into TTAA chromosomal sites. The piggyBac transposon system has no payload limit for the genes of interest that can be included between the ITRs. In embodiments, the transposon is a piggyBac transposon or a piggyBac-like transposon.

[00340] Examples of piggyBac and piggyBac-like transposases and transposons include, for example, those disclosed in WO2019/046815, the contents of which are incorporated herein by reference in their entirety. In embodiments, the piggyBac or piggyBac-like transposase is hyperactive. A hyperactive piggyBac or piggyBac-like transposase is a transposase that is more active than the naturally occurring variant from which it is derived. In embodiments, the hyperactive piggyBac or piggyBac-like transposase enzyme is isolated or derived from Bombyx mori. A list of hyperactive amino acid substitutions can be found in US patent No. 10,041,077, the contents of which are incorporated herein by reference in their entirety. In embodiments, the piggyBac or piggyBac-like transposase is integration deficient. In embodiments, an integration deficient piggyBac or piggyBac-like transposase is a transposase that can excise its corresponding transposon, but that integrates the excised transposon at a lower frequency than a corresponding wild-type transposase. A list of integration deficient amino acid substitutions can be found in US patent No. 10,041,077, the contents of which are incorporated by reference in their entirety.

[00341] In embodiments, the piggyBac or piggyBac-like transposon is capable of insertion by a piggyBac or piggyBac-like transposase at the sequence 5'-TTAT-3 within a target nucleic acid. In embodiments, and, in particular, embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac transposase. In embodiments, and, in particular, embodiments wherein the transposon is a piggyBac- like transposon, the transposase is a piggyBac-like transposase. In embodiments, and, in particular, embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac™ or a Super piggyBac™ (SPB) transposase. In embodiments, and, in particular, embodiments wherein the transposase is a Super piggyBac™ (SPB) transposase, the sequence encoding the transposase is an mRNA sequence.

[00342] The sleeping beauty (SB) transposon is transposed into the target genome by the Sleeping Beauty transposase that recognizes ITRs, and moves the contents between the ITRs into TA chromosomal sites. In embodiments, the transposon is a Sleeping Beauty transposon. In embodiments, the transposase enzyme is a Sleeping Beauty transposase enzyme (see, for example, US Patent No. 9,228,180, the contents of which are incorporated herein in their entirety). In embodiments, the Sleeping Beauty transposase is a hyperactive Sleeping Beauty (SB100X) transposase.

[00343] The Helraiser transposon is transposed by the Helitron transposase. Unlike other transposases, the Helitron transposase does not contain an RNase-H like catalytic domain, but instead comprises a RepHel motif made up of a replication initiator domain (Rep) and a DNA helicase domain. The Rep domain is a nuclease domain of the HUH superfamily of nucleases. In embodiments, the transposon is a Helraiser transposon. In embodiments of the Helraiser transposon sequence, the transposase is flanked by left and right terminal sequences termed LTS and RTS. In embodiments, these sequences terminate with a conserved 5'-TC/CTAG-3' motif. In embodiments, a 19 bp palindromic sequence with the potential to form the hairpin termination structure is located 11 nucleotides upstream of the RTS and comprises the sequence GTGCACGAATTTCGTGCACCGGGCCACTAG. In embodiments, and, in particular embodiments wherein the transposon is a Helraiser transposon, the transposase enzyme is a Helitron transposase enzyme.

[00344] Tol2 transposons may be isolated or derived from the genome of the medaka fish, and may be similar to transposons of the hAT family. Exemplary Tol2 transposons of the disclosure are encoded by a sequence comprising about 4.7 kilobases and contain a gene encoding the Tol2 transposase, which contains four exons. In embodiments, the transposon is a Tol2 transposon. In certain embodiments of the methods of the disclosure, and, in particular those embodiments wherein the transposon is a Tol2 transposon, the transposase enzyme is a Tol2 transposase enzyme.

[00345] In embodiments, a vector comprises the recombinant and non-naturally occurring DNA sequence encoding the transposon. In embodiments, the vector comprises any form of DNA and wherein the vector comprises at least 100 nucleotides (nts), 500 nts, 1000 nts, 1500 nts, 2000 nts, 2500 nts, 3000 nts, 3500 nts, 4000 nts, 4500 nts, 5000 nts, 6500 nts, 7000 nts, 7500 nts, 8000 nts, 8500 nts, 9000 nts, 9500 nts, 10,000 nts or any number of nucleotides in between. In embodiments, the vector comprises single-stranded or double-stranded DNA. In embodiments, the vector comprises circular DNA. In embodiments, the vector is a plasmid vector, a nanoplasmid vector, a minicircle. In

embodiments, the vector comprises linear or linearized DNA. In embodiments, the vector is a double-stranded doggybone™ DNA sequence.

[00346] In embodiments, the recombinant and non-naturally occurring DNA sequence encoding a transposon further comprises a sequence encoding one or more immune checkpoint genes.

[00347] In embodiments, the nucleic acid sequence encoding the transposase enzyme is a DNA sequence, and an amount of the DNA sequence encoding the transposase enzyme and an amount of the DNA sequence encoding the transposon is equal to or less than 10.0 μg per 100 μL, less than 7.5 μg per 100 μL, less than 6.0 μg per 100 μL, less than 5.0 μg per 100 μL, less than 2.5 μg per 100 μL, or less than 1.67 μg per 100 μL, less than 0.55 μg per 100 μL, less than 0.19 μg per 100 μL, less than 0.10 μg per 100 μL of an electroporation or nucleofection reaction. In certain embodiments, a concentration of the amount of the DNA sequence encoding the transposase enzyme and an amount of the DNA sequence encoding the transposon in the electroporation or nucleofection reaction is equal to or less than 100 μg/mL, equal to or less than 75 μg/mL, equal to or less than 60 μg/mL, equal to or less than 50 μg/mL, equal to or less than 25 μg/mL, equal to or less than 16.7 μg/mL, equal to or less than 5.5 μg/mL, equal to or less than 1.9 μg/mL, equal to or less than 1.0 μg/mL.

[00348] In embodiments, the nucleic acid sequence encoding the transposase enzyme is an RNA sequence, and an amount of the RNA sequence encoding the transposase enzyme and an amount of the RNA sequence encoding the transposon is equal to or less than 10.0 μg per 100 μL, less than 7.5 μg per 100 μL, less than 6.0 μg per 100 μL, less than 5.0 μg per 100 μL, less than 2.5 μg per 100 μL, or less than 1.67 μg per 100 μL, less than 0.55 μg per 100 μL, less than 0.19 μg per 100 μL, less than 0.10 μg per 100 μL of an electroporation or nucleofection reaction. In certain embodiments, a concentration of the amount of the RNA sequence encoding the transposase enzyme and an amount of the RNA sequence encoding the transposon in the electroporation or nucleofection reaction is equal to or less than 100 μg/mL, equal to or less than 75 μg/mL, equal to or less than 60 μg/mL, equal to or less than 50 μg/mL, equal to or less than 25 μg/mL, equal to or less than 16.7 μg/mL, equal to or less than 5.5 μg/mL, equal to or less than 1.9 μg/mL, equal to or less than 1.0 μg/mL.

[00349] In embodiments, the TILs are further modified by a second gene editing tool, including, but not limited to those described herein. In embodiments, the second gene editing tool may include an excision-only piggyBac transposase to re-excise the inserted sequences or any portion thereof. For example, the excision-only piggyBac transposase may be used to "re-excise" the transposon.

[00350] According to some embodiments, a piggyBac system comprises a transposon/transposase system, wherein the transposase recognizes the ITRs located on both sides of the transposon cassette comprising a cargo encoding one or more immune checkpoint genes, and excises the transposable element to integrate it into TT/AA chromosomal sites, resulting in genomic insertion of the transposon cassette and expression of the one or more immune checkpoint genes. According to some embodiments, the cargo encodes two or more immune checkpoint molecules.

[00351] Non-limiting examples of genes that may be enhanced by permanently gene-editing TILs via a piggyBac method include CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, IL-2, IL-4, IL-7, IL-10, IL-15, IL-18, IL-21, the NOTCH 1/2 intracellular domain (ICD), and/or the NOTCH ligand mDLL1.

[00352] Examples of systems, methods, and compositions for altering the expression of a target gene sequence by a piggyBac method, and which may be used in accordance with embodiments of the present invention, are described in WO2019/046815, WO2015006700, WO2010085699, WO2010099301, WO2010099296, WO2006122442, WO2001081565, and WO1998040510, the contents of which are incorporated herein by reference in their entirety.

[00353] Resources for carrying out piggyBac methods, such as plasmids for expressing transposons/transposases, are commercially available from companies such as Demeetra and Hera Biolabs.

[00354] According to some embodiments, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into multiple tumor fragments;

(b) adding the tumor fragments into a closed system;

(c) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 3 to 11 days to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area;

(d) stimulating the second population of TILs by adding OKT-3 and culturing for about 1 to 3 days, wherein the transition from step (c) to step (d) occurs without opening the system;

(e) sterile electroporating the second population of TILs to effect transfer of at least one gene editor into a plurality of cells in the second population of TILs;

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

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

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

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

(j) optionally cryopreserving the harvested TIL population using a cryopreservation medium,

wherein the electroporation step comprises the delivery of at least one gene editor system comprising a piggyBac system, which at least one gene editor system effects expression of at least one immunomodulatory composition at the cell surface of and modulates expression of at least one checkpoint protein in the plurality of cells of the second population of TILs.

[00355] According to some embodiments, a method for expanding tumor infiltrating lymphocytes (TILs) into a therapeutic population of TILs comprises:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into multiple tumor fragments;

(b) adding the tumor fragments into a closed system;

(c) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2 and optionally comprising OKT-3 and/or a 4-1BB agonist antibody for about 3 to 11 days to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area;

(d) stimulating the second population of TILs by adding OKT-3 and culturing for about 1 to 3 days to obtain the second population of TILs, wherein the transition from step (c) to step (d) occurs without opening the system;

(e) sterile electroporating the second population of TILs to effect transfer of at least one gene editor into a plurality of cells in the second population of TILs;

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

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

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

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

(j) optionally cryopreserving the harvested TIL population using a cryopreservation medium,

wherein the electroporation step comprises the delivery of at least one gene editor system comprising a piggyBac system, which at least one gene editor system effects expression of at least one immunomodulatory composition at the cell surface of in the plurality of cells of the second population of TILs. In some embodiments, the at least one immunomodulatory composition comprises an immunomodulatory agent fused to a membrane anchor (e.g., a membrane anchored immunomodulatory fusion protein described herein). In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-2, IL-7, IL-10, IL-12, IL-15, IL-18, IL-21, and a CD40 agonist (e.g., a CD40L or an agonistic CD40 binding domain). In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-2, IL-12, IL-15, IL-18, IL-21, and a CD40 agonist. In some embodiments, the immunomodulatory agent is selected from the group consisting of IL-12, IL-15, IL-18, IL-21, and a CD40 agonist.

[00356] In some embodiments, a method of genetically modifying a population of TILs includes the use of a non-viral technique such as a piggyBac method (e.g., piggyBac transposons and transposases or piggyBac-like transposons and transposases). In some embodiments, the method comprises delivering to the TILs: (a) a nucleic acid or amino acid sequence comprising a sequence encoding a transposase enzyme; and (b) a recombinant and non-naturally occurring DNA sequence comprising a DNA sequence encoding a transposon. In certain embodiments of the methods of the disclosure, the sequence encoding a transposase enzyme is an mRNA sequence. The mRNA sequence encoding a transposase enzyme may be produced in vitro. In certain embodiments of the methods of the disclosure, the sequence encoding a transposase enzyme is a DNA sequence. The DNA sequence encoding a transposase enzyme may be produced in vitro. The DNA sequence may be a cDNA sequence. In certain embodiments of the methods of the disclosure, the sequence encoding a transposase enzyme is an amino acid sequence. The amino acid sequence encoding a transposase enzyme may be produced in vitro. A protein Super piggybac transposase (SPB) may be delivered following pre-incubation with transposon DNA. In certain embodiments, the transposon is a piggyBac transposon or a piggyBac-like transposon. In certain embodiments, and, in particular, those embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac transposase. In certain embodiments, and, in particular, those embodiments wherein the transposon is a piggyBac- like transposon, the transposase is a piggyBac-like transposase. In certain embodiments, the piggyBac transposase comprises an amino acid sequence comprising SEQ ID NO: 14487 of WO2019046815. In certain embodiments, and, in particular, those embodiments wherein the transposon is a piggyBac transposon, the transposase is a piggyBac™ or a Super piggyBac™ (SPB) transposase. In certain embodiments, and, in particular, those embodiments wherein the transposase is a Super piggyBac™ (SPB) transposase, the sequence encoding the transposase is an mRNA sequence. In certain embodiments of the methods of the disclosure, the transposase enzyme is a piggyBac™ (PB) transposase enzyme. The piggyBac (PB) transposase enzyme may comprise or consist of an amino acid sequence at least 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between identical to:

1 MGSSLDDEHI LSALLQSDDE LVGEDSDSEI SDHVSEDDVQ SDTEEAFIDE VHEVQPTSSG

61 SEILDEQNVI EQPGSSLASN RILTLPQRTI RGKNKHCWST SKSTRRSRVS ALNIVRSQRG

121 PTRMCRNIYD PLLCFKLFFT DEIISEIVKW TNAEISLKRR ESMTGATFRD TNEDEIYAFF

181 GILVMTAVRK DNHMSTDDLF DRSLSMVYVS VMSRDRFDFL IRCLRMDDKS IRPTLRENDV

241 FTPVRKIWDL FIHQCIQNYT PGAHLTIDEQ LLGFRGRCPF RMYIPNKPSK YGIKILMMCD

301 SGTKYMINGM PYLGRGTQTN GVPLGEYYVK ELSKPVHGSC RNITCDNWFT SIPLAKNLLQ

361 EPYKLTIVGT VRSNKREIPE VLKNSRSRPV GTSMFCFDGP LTLVSYKPKP AKMVYLLSSC

421 DEDASINEST GKPQMVMYYN QTKGGVDTLD QMCSVMTCSR KTNRWPMALL YGMINIACIN

481 SFIIYSHNVS SKGEKVQSRK KFMRNLYMSL TSSFMRKRLE APTLKRYLRD NISNILPNEV

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

[00357] In certain embodiments of the methods of the disclosure, the transposon is a Sleeping Beauty transposon. In certain embodiments of the methods of the disclosure, the transposase enzyme is a Sleeping Beauty transposase enzyme (see, for example, US Patent No. 9,228,180, the contents of which are incorporated herein in their entirety). In certain embodiments, the Sleeping Beauty transposase is a hyperactive Sleeping Beauty (SB100X) transposase. In certain embodiments, the Sleeping Beauty transposase enzyme comprises an amino acid sequence at least 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between identical to:

1 MGKSKEISQD LRKKIVDLHK SGSSLGAISK RLKVPRSSVQ TIVRKYKHHG TTQPSYRSGR

61 RRVLSPRDER TLVRKVQINP RTTAKDLVKM LEETGTKVSI STVKRVLYRH NLKGRSARKK

121 PLLQNRHKKA RLRFATAHGD KDRTFWRNVL WSDETKIELF GHNDHRYVWR KKGEACKPKN

181 TIPTVKHGGG SIMLWGCFAA GGTGALHKID GIMRKENYVD ILKQHLKTSV RKLKLGRK V

241 FQMDNDPKHT SKWAKWLKD NKVKVLEWPS QSPDLNPIEN LWAELKKRVR ARRPTNLTQL

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

[00358] In certain embodiments, including those wherein the Sleeping Beauty transposase is a hyperactive Sleeping Beauty (SB100X) transposase, the Sleeping Beauty transposase enzyme comprises an amino acid sequence at least at least 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between identical to:

1 MGKSKEISQD LRKRIVDLHK SGSSLGAISK RLAVPRSSVQ TIVRKYKHHG TTQPSYRSGR

61 RRVLSPRDER TLVRKVQINP RTTAKDLVKM LEETGTKVSI STVKRVLYRH NLKGHSARKK

121 PLLQNRHKKA RLRFATAHGD KDRTFWRNVL WSDETKIELF GHNDHRYVWR KKGEACKPKN

181 TIPTVKHGGG SIMLWGCFAA GGTGALHKID GIMDAVQYVD ILKQHLKTSV RKLKLGRKWV

241 FQHDNDPKHT SKWAKWLKD NKVKVLEWPS QSPDLNPIEN LWAELKKRVR ARRPTNLTQL

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

V. Gen 2 TIL Manufacturing Processes

[00359] An exemplary family of TIL processes known as Gen 2 (also known as process 2A) containing some of these features is depicted in Figures 1 and 2. An embodiment of Gen 2 is shown in Figure 2.

[00360] 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.

[00361] 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.

[00362] In some embodiments, the first expansion (including processes referred to as the pre-REP as well as processes shown in Figure 1 and Figure 36 as Step B) is shortened to 3 to 14 days and the second expansion (including processes referred to as the REP as well as processes shown in Figure 1 or Figure 36 as Step D) is shorted to 7 to 14 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the first expansion (for example, an expansion described as Step B in Figure 1 or Figure 36) is shortened to 11 days and the second expansion (for example, an expansion as described in Step D in Figure 1 or Figure 36) is shortened to 11 days. In some embodiments, the combination of the first expansion and second expansion (for example, expansions described as Step B and Step D in Figure 1 or Figure 36) is shortened to 22 days, as discussed in detail below and in the examples and figures.

[00363] The "Step" Designations A, B, C, *etc.*, below are in reference to Figure 1 or Figure 36 and in reference to certain embodiments described herein. The ordering of the Steps below and in Figure 1 and Figure 36 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

[00364] 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.

[00365] 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 non-small cell lung carcinoma (NSCLC). The solid tumor may be of skin tissue. In some embodiments, useful TILs are obtained from a melanoma.

[00366] Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 mm^3, with from about 2-3 mm^3 being particularly useful. 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_2, followed by repeated cycles of mechanical dissociation and incubation under the foregoing conditions until only small tissue

pieces are present. At the end of this process, if the cell suspension contains a large number of red blood cells or dead cells, a density gradient separation using FICOLL branched hydrophilic polysaccharide may be performed to remove these cells. Alternative methods known in the art may be used, such as those described in U.S. Patent Application Publication No. 2012/0244133 A1, the disclosure of which is incorporated by reference herein. Any of the foregoing methods may be used in any of the embodiments described herein for methods of expanding TILs or methods treating a cancer.

[00367] 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.

[00368] 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.

[00238] 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.

[00239] 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.

[00240] 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.

[00241] 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.

[00242] In some embodiment, the enzyme mixture includes about 10.2-ul of neutral protease (0.36 DMC U/mL), 21.3 µL of collagenase (1.2 PZ/mL) and 250-ul of DNAse I (200 U/mL) in about 4.7 mL of sterile HBSS.

[00369] 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 in an enzyme mixture comprising collagenase, DNase, and hyaluronidase. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% CO_2. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% CO_2 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_2 with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[00370] In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and neutral protease. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and neutral protease for 1-2 hours. 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_2. 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_2 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_2 with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

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

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

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

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

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

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

[00377] In general, the harvested cell suspension is called a "primary cell population" or a "freshly harvested" cell population.

[00378] 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.

[00379] In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained in, for example, Step A (as provided in Figure 1 or Figure 36). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the

fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3. In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 mm^3 to about 1500 mm^3. In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3. In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams. In some embodiments, the multiple fragments comprise about 4 fragments.

[00380] In some embodiments, the TILs are obtained from tumor fragments. In some embodiments, the tumor fragment is obtained by sharp dissection. In some embodiments, the tumor fragment is between about 1 mm^3 and 10 mm^3. In some embodiments, the tumor fragment is between about 1 mm^3 and 8 mm^3. In some embodiments, the tumor fragment is about 1 mm^3. In some embodiments, the tumor fragment is about 2 mm^3. In some embodiments, the tumor fragment is about 3 mm^3. In some embodiments, the tumor fragment is about 4 mm^3. In some embodiments, the tumor fragment is about 5 mm^3. In some embodiments, the tumor fragment is about 6 mm^3. In some embodiments, the tumor fragment is about 7 mm^3. In some embodiments, the tumor fragment is about 8 mm^3. In some embodiments, the tumor fragment is about 9 mm^3. In some embodiments, the tumor fragment is about 10 mm^3. 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.

[00381] 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.

[00382] 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_2 and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% CO_2, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% CO_2. In some embodiments, at the end of the final incubation if the cell suspension contains a large number of red blood cells or dead cells, a density gradient separation using Ficoll can be performed to remove these cells.

[00383] 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.

[00384] In some embodiments, cells can be optionally frozen after sample harvest and stored frozen prior to entry into the expansion described in Step B, which is described in further detail below, as well as exemplified in Figure 1 and Figure 36, as well as Figure 8.

1. Pleural effusion T-cells and TILs

[0001] 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.

[0002] 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 matter 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.

[0003] In some embodiments, the pleural fluid is in unprocessed form, directly as removed from the patient. In some embodiments, the unprocessed pleural fluid is placed in a standard blood collection tube, such as an EDTA or Heparin tube, prior to further processing steps. In some embodiments, the unprocessed pleural 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 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.

[0004] In some embodiments, the pleural fluid sample from the chosen subject may be diluted. In some embodiments, the dilution is 1:10 pleural fluid to diluent. In other embodiments, the dilution is 1:9 pleural fluid to diluent. In other embodiments, the dilution is 1:8 pleural fluid to diluent. In other embodiments, the dilution is 1:5 pleural fluid to diluent. In other embodiments, the dilution is 1:2 pleural fluid to diluent. In other embodiments, the dilution is 1:1 pleural fluid to diluent. In some embodiments, diluents include saline, phosphate buffered saline, another buffer or a physiologically acceptable diluent. In some embodiments, the sample is placed in the CellSave tube immediately after collection from the patient and dilution to avoid a decrease in the viable TILs, which may occur to a significant extent within 24-48 hours, if left in the untreated pleural fluid, even at 4°C. In some embodiments, the pleural fluid sample is placed in the appropriate collection tube within 1 hour, 5 hours, 10 hours, 15 hours, 24 hours, 36 hours, up to 48 hours after removal from the patient, and

dilution. In some embodiments, the pleural fluid sample is placed in the appropriate collection tube within 1 hour, 5 hours, 10 hours, 15 hours, 24 hours, 36 hours, up to 48 hours after removal from the patient, and dilution at 4°C.

[0005] In still other embodiments, pleural fluid samples are concentrated by conventional means prior to further processing steps. In some embodiments, this pre-treatment of the pleural fluid is preferable in circumstances in which the pleural fluid must be cryopreserved for shipment to a laboratory performing the method or for later analysis (*e.g.*, later than 24-48 hours post-collection). In some embodiments, the pleural fluid sample is prepared by centrifuging the pleural fluid sample after its withdrawal from the subject and resuspending the centrifugate or pellet in buffer. In some embodiments, the pleural fluid sample is subjected to multiple centrifugations and resuspensions, before it is cryopreserved for transport or later analysis and/or processing.

[0006] In some embodiments, pleural fluid samples are concentrated prior to further processing steps by using a filtration method. In some embodiments, the pleural fluid sample used in 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 fluid through the membrane but retains the tumor cells. In some embodiments, the diameter of the pores in the membrane may be at least 4 μM. In other embodiments the pore diameter may be 5 μM or more, and in other embodiment, any of 6, 7, 8, 9, or 10 μM. After filtration, the cells, including TILs, retained by the membrane may be rinsed off the membrane into a suitable physiologically acceptable buffer. Cells, including TILs, concentrated in this way may then be used in the further processing steps of the method.

[0007] In some embodiments, pleural fluid sample (including, for example, the untreated pleural fluid), diluted pleural fluid, or the resuspended cell pellet, is contacted with a lytic reagent that differentially lyses non-nucleated red blood cells present in the sample. In some embodiments, this step is performed prior to further processing steps in circumstances in which the pleural fluid contains substantial numbers of RBCs. Suitable lysing reagents include a single lytic reagent or a lytic reagent and a quench reagent, or a lytic agent, a quench reagent and a fixation reagent. Suitable lytic systems are marketed commercially and include the BD Pharm Lyse™ system (Becton Dickenson). Other lytic systems include the Versalyse™ system, the FACSlyse™ system (Becton Dickenson), the Immunoprep™ system or Erythrolyse II system (Beckman Coulter, Inc.), or an ammonium chloride system. In some embodiments, the lytic reagent can vary with the primary requirements being efficient lysis of the red blood cells, and the conservation of the TILs and phenotypic properties of the TILs in the pleural fluid. In addition to

employing a single reagent for lysis, the lytic systems useful in methods described herein can include a second reagent, *e.g.*, one that quenches or retards the effect of the lytic reagent during the remaining steps of the method, *e.g.*, Stabilyse™ reagent (Beckman Coulter, Inc.). A conventional fixation reagent may also be employed depending upon the choice of lytic reagents or the preferred implementation of the method.

[0008] In some embodiments, the pleural fluid sample, unprocessed, diluted or multiply centrifuged or processed as described herein above is cryopreserved at a temperature of about −140°C prior to being further processed and/or expanded as provided herein.

B. STEP B: First Expansion

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.

[0009] The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1 or Figure 36. 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 is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCRα/β).

[0010] After dissection or digestion of tumor fragments, for example such as described in Step A of Figure 1 or Figure 36, the resulting cells are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 3 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 7 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 10 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of about 11 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells.

[0011] 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 1 or Figure 36, 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.

[0012] In embodiments where TIL cultures are initiated in 24-well plates, for example, using Costar 24-well cell culture cluster, flat bottom (Corning Incorporated, Corning, NY, each well can be seeded with 1×10^6 tumor digest cells or one tumor fragment in 2 mL of complete medium (CM) with IL-2 (6000 IU/mL; Chiron Corp., Emeryville, CA). In some embodiments, the tumor fragment is between about 1 mm^3 and 10 mm^3.

[0013] In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, CM for Step B consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10 cm^2 gas-permeable silicon bottom (for example, G-REX10; Wilson Wolf Manufacturing, New Brighton, MN), each flask was loaded with 10–40 $\times 10^6$ viable tumor digest cells or 5–30 tumor fragments in 10–40 mL of CM with IL-2. Both the G-REX10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% CO_2 and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days.

[0014] 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 1×10^8 bulk TIL cells. In some embodiments, the growth media during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the IL is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of $20\text{-}30\times10^6$ IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 20×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 25×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 30×10^6 IU/mg for a 1 mg vial. In some embodiments, the IL- 2 stock solution has a final concentration of $4\text{-}8\times10^6$ IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of $5\text{-}7\times10^6$ IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of 6×10^6 IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepare as described in Example 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.

[0015] 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.

[0016] 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.

[0017] 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.

[0018] In some embodiments, OKT-3 is present in the cell culture medium at the initiation (day 0) of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium at any time during the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 1 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 2 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 3 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 4 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 5 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 6 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 7 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 8 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 9 of the first

expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 10 of the first expansion. In some embodiments, OKT-3 is added to the cell culture medium on day 11 of the first expansion.

[0019] In some embodiments, OKT-3 is added at one or more times between day 0 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 0 and day 3 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 0 and day 2 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 0 and day 1 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 1 and day 2 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 1 and day 3 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 2 and day 3 of the first expansion.

[0020] In some embodiments, OKT-3 is added at one or more times between day 1 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 2 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 3 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 4 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 5 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 6 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 7 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 8 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 9 and day 11 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 10 and day 11 of the first expansion.

[0021] In some embodiments, OKT-3 is added at one or more times between day 0 and day 7 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 0 and day 6 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 0 and day 5 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 0 and day 4 of the first expansion.

[0022] In some embodiments, OKT-3 is added at one or more times between day 1 and day 7 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 1 and day 6 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 1 and day

5 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 1 and day 4 of the first expansion.

[0023] In some embodiments, OKT-3 is added at one or more times between day 2 and day 7 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 2 and day 6 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 2 and day 5 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 2 and day 4 of the first expansion.

[0024] In some embodiments, OKT-3 is added at one or more times between day 3 and day 7 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 3 and day 6 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 3 and day 5 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 3 and day 4 of the first expansion.

[0025] In some embodiments, OKT-3 is added at one or more times between day 4 and day 7 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 4 and day 6 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 4 and day 5 of the first expansion.

[0026] In some embodiments, OKT-3 is added at one or more times between day 5 and day 7 of the first expansion. In some embodiments, OKT-3 is added at one or more times between day 5 and day 6 of the first expansion.

[0027] In some embodiments, OKT-3 is added at one or more times between day 6 and day 7 of the first expansion.

[0028] 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.

[0029] 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.

[0030] In some embodiments, the first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, it is referred to as CM1 (culture medium 1). In some embodiments, CM consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In embodiments where cultures are initiated in gas-permeable flasks with a 40 mL capacity and a 10cm^2 gas-permeable silicon bottom (for example, G-REX10; Wilson Wolf Manufacturing, New Brighton, MN), each flask was loaded with 10–40x10^6 viable tumor digest cells or 5–30 tumor fragments in 10–40mL of CM with IL-2. Both the G-REX10 and 24-well plates were incubated in a humidified incubator at 37°C in 5% CO_2 and 5 days after culture initiation, half the media was removed and replaced with fresh CM and IL-2 and after day 5, half the media was changed every 2–3 days. In some embodiments, the CM is the CM1 described in the Examples, *see*, Example 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.

[0031] In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1 or Figure 36, 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 1 or Figure 36, which can include those sometimes referred to as the pre-REP) is shortened to 7 to 14 days, as discussed in the Examples and shown in Figures 4 and 5, as well as including for example, an expansion as described in Step B of Figure 1 or Figure 36. In some embodiments, the first expansion of Step B is shortened to 10-14 days. In some embodiments, the first expansion is shortened to 11 days, as discussed in, for example, an expansion as described in Step B of Figure 1 or Figure 36.

[0032] 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.

[0033] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first expansion, including for example during a Step B processes according to Figure 1 or Figure 36, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 1 or Figure 36 and as described herein.

[0034] In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 1 or Figure 36) 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.

[0035] In some embodiments, the first expansion, for example, Step B according to Figure 1 or Figure 36, 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

[0036] 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.
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.

[00243] 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**

[0037] In some cases, the bulk TIL population obtained from the first expansion, including for example the TIL population obtained from for example, Step B as indicated in Figure 1 or Figure 36, 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.

[0038] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1 or Figure 36) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1 or Figure 36) 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.

[0039] 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.

[0040] In some embodiments, the TILs are not stored after the first expansion and prior to the second expansion, and the TILs proceed directly to the second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 1 or Figure 36). In some embodiments, the transition occurs in a 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.

[0041] In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 1 or Figure 36, 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.

[0042] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 36A-D or Figure 8I-P) are transitioned to an activation or a gene-editing step. In some embodiments, the transition from the first expansion to the activation or gene-editing step 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 activation or gene-editing step occurs at about 3 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 13 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 12 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 9 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 8 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 7 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 6 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 5 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 3 days to 4 days from when fragmentation occurs. In some embodiments, the transition from the

first expansion to the activation or gene-editing step occurs at about 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 13 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 12 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 9 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 8 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 7 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 6 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 4 days to 5 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 13 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 12 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 9 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 8 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 7 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 5 days to 6 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or

gene-editing step occurs at about 6 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 13 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 12 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 11 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 9 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 8 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs at about 6 days to 7 days from when fragmentation occurs.

[0043] In some embodiments, the transition from the first expansion to the activation or gene-editing step occurs in a closed system, as described herein.

[0044] In some embodiments, the activation step comprises culturing the TILs in medium comprising anti-CD3 and/or anti-CD38 beads for about 1-7 days. In some embodiments, the activation step comprises culturing the TILs in medium comprising anti-CD3 beads for about 1-7 days. In some embodiments, the activation step comprises culturing the TILs in medium comprising anti-CD38 beads for about 1-7 days. In some embodiments, the activation step comprises culturing the TILs in medium comprising anti-CD3 and anti-CD38 beads for about 1-7 days. In some embodiments, the activation step comprises culturing the TILs in medium comprising OKT3 for about 1-7 days. In some embodiments, the activation step is performed for about 1-7 days. In some embodiments, the activation step is performed for about 1-7 days, about 2-7 days, about 3-7 days, about 4-7 days, about 5-7 days, about 6-7 days, about 1-6 days, about 2-6 days, about 3-6 days, about 4-6 days, about 5-6 days, about 1-5 days, about 2-5 days, about 3-5 days, about 4-5 days, about 1-4, days, about 2-4, days, about 3-4, days, about 1-3 days, about 2-3 days, or about 1-2 days. In some embodiments, the activation step is performed for about 1 day. In some embodiments, the activation step is performed for about 2 days. In some embodiments, the activation step is performed for about 3 days. In some embodiments, the activation step is performed for about 4 days. In some embodiments, the activation step is performed for about 5 days. In

some embodiments, the activation step is performed for about 6 days. In some embodiments, the activation step is performed for about 7 days.

[0045] Any suitable anti-CD3/anti-CD38 beads known to those in the art in view of the instant specification can be used. Suitable anti-CD3/anti-CD38 beads include, but are not limited to, commercially available products including, but not limited to, Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (available commercially from Invitrogen), ImmunoCult™ Human CD3/CD28 T Cell Activator (available commercially from StemCell Technologies), and T Cell TransAct™ (available commercially from Miltenyi Biotec).

[0046] In some embodiments, the activation step is optional. In some embodiments, the activation step is optional if the first expansion included OKT-3.

[0047] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 36C-D) or from the activation step (for example, from Step C as indicated in Figure 36A-B) are transitioned to a gene-editing step.

[0048] In some embodiments, the gene-editing step comprises performing a sterile electroporation step on the population of TILs. In some embodiments, the sterile electroporation step mediates the transfer of at least one gene editor. According to some embodiments, the gene editor is a TALE nuclease system for modulating the expression of at least one protein. According to some embodiments, the TALE nuclease system downmodulates expression of PD-1. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of CTLA-4. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of LAG-3. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of CISH. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of TIGIT. According to some embodiments, the gene editor further comprises a TALE nuclease system that downmodulates expression of CBL-B. According to some embodiments, the resulting TILs are PD-1 knockout TILs. According to some embodiments, the resulting TILs are CTLA-4 knockout TILs. According to some embodiments, the resulting TILs are LAG-3 knockout TILs. According to some embodiments, the resulting TILs are CISH knockout TILs. According to some embodiments, the resulting TILs are CBL-B knockout TILs. According to some embodiments, the resulting TILs are TIGIT knockout TILs. According to some embodiments, the resulting TILs exhibit downmodulated expression of PD-1 and downmodulated

expression of one or more of CTLA-4, LAG-3, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CTLA-4 and downmodulated expression of one or more of PD-1, LAG-3, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of LAG-3 and downmodulated expression of one or more of PD-1, CTLA-4, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CISH and downmodulated expression of one or more of PD-1, LAG-3, CTLA-4, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CBL-B and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, TIGIT and PD-1. According to some embodiments, the resulting TILs exhibit downmodulated expression of TIGIT and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, CBL-B and PD-1. According to some embodiments, the resulting TILs are PD-1/CTLA-4 double knockout TILs. According to some embodiments, the resulting TILs are PD-1/LAG-3 double knockout TILs. According to some embodiments, the resulting TILs are PD-1/CISH double knockout TILs. According to some embodiments, the resulting TILs are PD-1/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are PD-1/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/LAG-3 double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/CISH double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/CISH double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CISH/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are CISH/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CBL-B/TIGIT double knockout TILs.

[0049] In some embodiments, the TILs obtained from the gene-editing step or the resting step (for example, from Step C as indicated in Figure 36A-D or Figure 8I-P) are transitioned to a second electroporation step. In some embodiments, there is a resting step between the first and second electroporation step. In some embodiments, the resting step is carried out at about 30°C, about 30.5°C, about 31°C, about 31.5°C, about 32°C, about 32.5°C, about 33°C, about 33.5°C, about 34°C, about 34.5°C, about 35°C, about 35.5°C, about 36°C, about 36.5°C, about 37°C, about 37.5°C, about 38°C, about 38.5°C, about 39°C, about 39.5°C, about 40°C. According to some embodiments, the resting step

is carried out 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 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 1.5 days, about 2 days, about 2.5 days, or about 3 days. In some embodiments, the second electroporation step mediates the transfer of at least one gene editor. According to some embodiments, the gene editor is a TALE nuclease system for modulating the expression of at least one protein. According to some embodiments, the TALE nuclease system downmodulates expression of PD-1. According to some embodiments, the TALE nuclease system downmodulates CTLA-4. According to some embodiments, the TALE nuclease system downmodulates expression of CISH. According to some embodiments, the TALE nuclease system downmodulates expression of LAG-3. According to some embodiments, the TALE nuclease system downmodulates expression of TIGIT. According to some embodiments, the TALE nuclease system downmodulates expression of CBL-B. According to some embodiments, the resulting TILs are PD-1 knockout TILs. According to some embodiments, the resulting TILs are CTLA-4 knockout TILs. According to some embodiments, the resulting TILs are LAG-3 knockout TILs. According to some embodiments, the resulting TILs are CISH knockout TILs. According to some embodiments, the resulting TILs are CBL-B knockout TILs. According to some embodiments, the resulting TILs are TIGIT knockout TILs. According to some embodiments, the resulting TILs exhibit downmodulated expression of PD-1 and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CTLA-4 and downmodulated expression of one or more of PD-1, LAG-3, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of LAG-3 and downmodulated expression of one or more of PD-1, CTLA-4, CISH, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CISH and downmodulated expression of one or more of PD-1, LAG-3, CTLA-4, TIGIT and CBL-B. According to some embodiments, the resulting TILs exhibit downmodulated expression of CBL-B and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, TIGIT and PD-1. According to some embodiments, the resulting TILs exhibit downmodulated expression of TIGIT and downmodulated expression of one or more of CTLA-4, LAG-3, CISH, CBL-B and PD-1. According to some embodiments, the resulting TILs are PD-1/CTLA-4 double knockout TILs. According to some embodiments, the resulting TILs are PD-1/LAG-3 double knockout TILs. According to some embodiments, the resulting TILs are PD-1/CISH double knockout TILs. According to some embodiments, the resulting TILs are PD-1/CBL-B double knockout TILs. According to some embodiments, the resulting

TILs are PD-1/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/LAG-3 double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/CISH double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are CTLA-4/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/CISH double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are LAG-3/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CISH/CBL-B double knockout TILs. According to some embodiments, the resulting TILs are CISH/TIGIT double knockout TILs. According to some embodiments, the resulting TILs are CBL-B/TIGIT double knockout TILs.

[0050] In some embodiments, the step of gene-editing further comprises a resting step. According to some embodiments, the resting step comprises incubating the fourth population of TILs at about 30-40 °C with about 5% CO_2. According to some embodiments, the resting step is carried out at about 30°C, about 30.5°C, about 31°C, about 31.5°C, about 32°C, about 32.5°C, about 33°C, about 33.5°C, about 34°C, about 34.5°C, about 35°C, about 35.5°C, about 36°C, about 36.5°C, about 37°C, about 37.5°C, about 38°C, about 38.5°C, about 39°C, about 39.5°C, about 40°C. According to some embodiments, the resting step is carried out 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 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about 15 hours to about 23 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 15 hours to about 23 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 15 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 16 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth

population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 17 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 18 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 19 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 20 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 21 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 22 hours at about 30°C. According to some embodiments, the resting step comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 23 hours at about 30°C.

[0051] In some embodiments, the TILs obtained from the gene-editing step or the resting step (for example, from Step C as indicated in Figure 36A-D or Figure 8I-P) are transitioned to a second expansion step (for example, from Step D as indicated in Figure 36A-D or Figure 8I-P). In some embodiments, the transition from the gene-editing step or the resting step to the second expansion step occurs at about 0.5 days, 1 day, 2 days, 3 days, or 4 days from the gene-editing step or the resting step.

[0052] In some embodiments, the transition from the gene-editing step or the resting step to the second expansion step occurs in a closed system, as described herein.

[0053] In some embodiments, the TILs are not stored after the gene-editing step or the resting step 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 C to Step D as shown in Figure 36A-D or Figure 8I-P).

[0054] In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 36, 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

[0055] In some embodiments, the TIL cell population is expanded in number after harvest and initial bulk processing for example, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 1 or Figure 36). This further expansion is referred to herein as the second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (REP); as well as processes as indicated in Step D of Figure 1 or Figure 36. 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.

[0056] In some embodiments, the second expansion or second TIL expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 1 or Figure 36) 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.

[0057] 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 1 or Figure 36). For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/mL of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San Diego, CA, USA). TILs can be expanded to induce further stimulation of

the TILs *in vitro* by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 μM MART-1 :26-35 (27 L) or gpl 00:209-217 (210M), optionally in the presence of a T-cell growth factor, such as 300 IU/mL IL-2 or IL-15. Other suitable antigens may include, *e.g.*, NY-ESO-1, TRP-1, TRP-2, tyrosinase cancer antigen, MAGE-A3, SSX-2, and VEGFR2, or antigenic portions thereof. TIL may also be rapidly expanded by re-stimulation with the same antigen(s) of the cancer pulsed onto HLA-A2-expressing antigen-presenting cells. Alternatively, the TILs can be further re-stimulated with, *e.g.*, example, irradiated, autologous lymphocytes or with irradiated HLA-A2+ allogeneic lymphocytes and IL-2. In some embodiments, the re-stimulation occurs as part of the second expansion. In some embodiments, the second expansion occurs in the presence of irradiated, autologous lymphocytes or with irradiated HLA-A2+ allogeneic lymphocytes and IL-2.

[0058] 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.

[0059] 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.

[0060] 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.

[0061] 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.

[0062] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the second expansion, including for example during a Step D processes according to Figure 1 or Figure 36, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step D processes according to Figure 1 or Figure 36 and as described herein.

[0063] 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).

[0064] 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.

[0065] 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.

[0066] 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.

[0067] 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.

[0068] 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.

[0069] 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 x 10^6 TILs suspended in 150 mL of media may be added to each T-175 flask. The TILs may be cultured in a 1 to 1 mixture of CM and AIM-V medium, supplemented with 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3. The T-175 flasks may be incubated at 37° C in 5% CO_2. Half the media may be exchanged on day 5 using 50/50 medium with 3000 IU per mL of IL-2. In some embodiments, on day 7 cells from two T-175 flasks may be combined in a 3 L bag and 300 mL of AIM V with 5% human AB serum and 3000 IU per mL of IL-2 was added to the 300 mL of TIL suspension. The number of cells in each bag was counted every day or two and fresh media was added to keep the cell count between 0.5 and 2.0 x 10^6 cells/mL.

[0070] 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 1 or Figure 36) may be performed in 500 mL capacity gas permeable flasks with 100 cm gas-permeable silicon bottoms (G-REX-100, commercially available from Wilson Wolf Manufacturing Corporation, New Brighton, MN, USA), 5 × 10^6 or 10 × 10^6 TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per mL of anti-CD3 (OKT3). The G-REX-100 flasks may be incubated at 37°C in 5% CO_2. On day 5, 250 mL of supernatant may be removed and placed into centrifuge bottles and

centrifuged at 1500 rpm (491 × g) for 10 minutes. The TIL pellets may be re-suspended with 150 mL of fresh medium with 5% human AB serum, 3000 IU per mL of IL-2, and added back to the original G-REX-100 flasks. When TIL are expanded serially in G-REX-100 flasks, on day 7 the TIL in each G-REX-100 may be suspended in the 300 mL of media present in each flask and the cell suspension may be divided into 3 100 mL aliquots that may be used to seed 3 G-REX-100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU per mL of IL-2 may be added to each flask. The G-REX-100 flasks may be incubated at 37° C in 5% CO_2 and after 4 days 150 mL of AIM-V with 3000 IU per mL of IL-2 may be added to each G-REX-100 flask. The cells may be harvested on day 14 of culture. In some embodiments, the process employed varying centrifugation speeds (400g, 300g, 200g for 5 minutes) and varying numbers of repetitions.

[0071] 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.

[0072] 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.

[0073] 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.

[0074] 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 1×10^6 TIL are suspended in about 150 mL of media and this is added to each T-175 flask. The TIL are cultured with irradiated (50 Gy) allogeneic PBMC as "feeder" cells at a ratio of 1 to 100 and the cells were cultured in a 1 to 1 mixture of CM and AIM-V medium (50/50 medium), supplemented with 3000 IU/mL of IL-2 and 30 ng/mL of anti-CD3. The T-175 flasks are incubated at 37°C in 5% CO_2. In some embodiments, half the media is changed on day 5 using 50/50 medium with 3000 IU/mL of IL-2. In some embodiments, on day 7, cells from 2 T-175 flasks are combined in a 3 L bag and 300 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to the 300 mL of TIL suspension. The number of cells in each bag can be counted every day or two and fresh media can be added to keep the cell count between about 0.5 and about 2.0×10^6 cells/mL.

[0075] In some embodiments, the second expansion (including expansions referred to as REP) are performed in 500 mL capacity flasks with 100 cm^2 gas-permeable silicon bottoms (G-REX-100, Wilson Wolf) about 5×10^6 or 10×10^6 TIL are cultured with irradiated allogeneic PBMC at a ratio of 1 to 100 in 400 mL of 50/50 medium, supplemented with 3000 IU/mL of IL-2 and 30 ng/ mL of anti-CD3. The G-REX-100 flasks are incubated at 37°C in 5% CO_2. 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 embodiments where TILs are expanded serially in G-REX-100 flasks, on day 7 the TIL in each G-REX-100 are suspended in the 300 mL of media present in each flask and the cell suspension was divided into three 100 mL aliquots that are used to seed 3 G-REX-100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to each flask. The G-REX-100 flasks are incubated at 37°C in 5% CO_2 and after 4 days 150 mL of AIM-V with 3000 IU/mL of IL-2 is added to each G-REX-100 flask. The cells are harvested on day 14 of culture. In some embodiments, the process employed varying centrifugation speeds (400g, 300g, 200g for 5 minutes) and varying numbers of repetitions.

[0076] The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained in the second expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin heavy chain. In some embodiments, the diversity is in the immunoglobulin is in the immunoglobulin light chain. In some embodiments, the diversity is in the T-cell receptor. In some embodiments, the diversity is in one of the T-cell receptors selected from the group consisting of alpha, beta, gamma, and delta receptors. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha and/or beta. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) alpha. In some embodiments, there is an increase in the expression of T-cell receptor (TCR) beta. In some embodiments, there is an increase in the expression of TCRab (*i.e.*, TCRα/β).

[0077] 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.

[0078] In some embodiments, the second expansion, for example, Step D according to Figure 1 or Figure 36, 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.

[0079] 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.

[0080] 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.

[0081] In some embodiments, the first small scale TIL culture is apportioned into a plurality of about 2 to 5 subpopulations of TILs.

[0082] 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.

[0083] 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.

[0084] 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.

[0085] 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.

[0086] 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.

[0087] 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.

[0088] 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.

[0089] 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.

[0090] 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.

[0091] 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.

[0092] 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.

[0093] 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.

[0094] In some embodiments, the splitting of the rapid expansion occurs in a closed system.

[0095] 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

[0096] In some embodiments, the second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 1 or Figure 36, 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.

[0097] 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.

[0098] 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).

[0099] 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.

[00100] 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.

[00101] 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.

[00102] In some embodiments, the second expansion procedures described herein require a ratio of about $2.5x10^9$ feeder cells to about $100x10^6$ TIL. In other embodiments, the second expansion procedures described herein require a ratio of about $2.5x10^9$ feeder cells to about $50x10^6$ TIL. In yet other embodiments, the second expansion procedures described herein require about $2.5x10^9$ feeder cells to about $25x10^6$ TIL.

[00103] 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.

[00104] 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.

[00105] 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

[00106] 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.

[00107] 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.

[00244] 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 I-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

[00108] After the second expansion step, cells can be harvested. In some embodiments the TILs are harvested after one, two, three, four or more expansion steps, for example as provided in Figure 1 or Figure 36. In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 1 or Figure 36.

[00109] 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.

[00110] 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.

[00111] In some embodiments, the harvest, for example, Step E according to Figure 1 or Figure 36, 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.

[00112] In some embodiments, Step E according to Figure 1 or Figure 36, 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.

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

[00113] After Steps A through E as provided in an exemplary order in Figure 1 or Figure 36 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.

[00114] 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.

VI. Gen 3 TIL Manufacturing Processes

[00115] Without being limited to any particular theory, it is believed that the priming first expansion that primes an activation of T cells followed by the rapid second expansion that boosts the activation of T cells as described in the methods of the invention allows the preparation of expanded T cells that retain a "younger" phenotype, and as such the expanded T cells of the invention are expected to exhibit greater cytotoxicity against cancer cells than T cells expanded by other methods. In particular, it is believed that an activation of T cells that is primed by exposure to an anti-CD3 antibody (*e.g.* OKT-3), IL-2 and optionally antigen-presenting cells (APCs) and then boosted by subsequent exposure to additional anti-CD-3 antibody (*e.g.* OKT-3), IL-2 and APCs as taught by the methods of the invention limits or avoids the maturation of T cells in culture, yielding a population of T cells with a less mature phenotype, which T cells are less exhausted by expansion in culture and exhibit greater cytotoxicity against cancer cells. In some embodiments, the step of rapid second expansion is split into a plurality of steps to achieve a scaling up of the culture by: (a) performing the rapid second expansion by culturing T cells in a small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period of about 3 to 4 days, and then (b) effecting the transfer of the T cells 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 T cells from the small scale culture in a larger scale culture in the second container for a period of about 4 to 7 days. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out of the culture by: (a) performing the rapid second expansion by culturing T cells 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 4 days, and then (b) effecting the transfer and apportioning of the T cells 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 T cells 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. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing T cells in a small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period

of about 3 to 4 days, and then (b) effecting the transfer and apportioning of the T cells 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 T cells 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. In some embodiments, the step of rapid expansion is split into a plurality of steps to achieve a scaling out and scaling up of the culture by: (a) performing the rapid second expansion by culturing T cells in a small scale culture in a first container, *e.g.*, a G-REX-100 MCS container, for a period of about 4 days, and then (b) effecting the transfer and apportioning of the T cells 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 T cells from the small scale culture transferred to such second container is cultured in a larger scale culture for a period of about 5 days.

[00116] In some embodiments, upon the splitting of the rapid expansion, each second container comprises at least 10^8 TILs. In some embodiments, upon the splitting of the rapid 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.

[00117] 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.

[00118] In some embodiments, after the completion of the rapid expansion, the plurality of subpopulations comprises a therapeutically effective amount of TILs. In some embodiments, after the completion of the rapid 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.

[00119] In some embodiments, the rapid expansion is performed for a period of about 1 to 5 days before being split into a plurality of steps. In some embodiments, the splitting of the rapid expansion occurs at about day 1, day 2, day 3, day 4, or day 5 after the initiation of the rapid expansion.

[00120] In some embodiments, the splitting of the rapid expansion occurs at about day 8, day 9, day 10, day 11, day 12, or day 13 after the initiation of the first expansion (*i.e.*, pre-REP expansion). In one

exemplary embodiment, the splitting of the rapid expansion occurs at about day 10 after the initiation of the priming first expansion. In another exemplary embodiment, the splitting of the rapid expansion occurs at about day 11 after the initiation of the priming first expansion.

[00121] In some embodiments, the rapid expansion is further performed for a period of about 4 to 11 days after the splitting. In some embodiments, the rapid expansion is further performed for a period of about 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, or 11 days after the splitting.

[00122] In some embodiments, the cell culture medium used for the rapid expansion before the splitting comprises the same components as the cell culture medium used for the rapid expansion after the splitting. In some embodiments, the cell culture medium used for the rapid expansion before the splitting comprises different components from the cell culture medium used for the rapid expansion after the splitting.

[00123] In some embodiments, the cell culture medium used for the rapid 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 expansion before the splitting comprises IL-2, OKT-3, and further optionally APCs. In some embodiments, the cell culture medium used for the rapid expansion before the splitting comprises IL-2, OKT-3 and APCs.

[00124] In some embodiments, the cell culture medium used for the rapid 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 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 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 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.

[00125] In some embodiments, the cell culture medium used for the rapid expansion after the splitting comprises IL-2, and optionally OKT-3. In some embodiments, the cell culture medium used for the rapid expansion after the splitting comprises IL-2, and OKT-3. In some embodiments, the cell culture medium used for the rapid expansion after the splitting is generated by replacing the cell culture medium used

for the rapid 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 expansion after the splitting is generated by replacing the cell culture medium used for the rapid expansion before the splitting with fresh culture medium comprising IL-2 and OKT-3.

[00126] In some embodiments, the splitting of the rapid expansion occurs in a closed system.

[00127] In some embodiments, the scaling up of the TIL culture during the rapid 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.

[00128] In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion begins to decrease, abate, decay or subside.

[00129] In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by at or about 1, 2, 3, 4, 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, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100%.

[00130] In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by a percentage in the range of at or about 1% to 100%.

[00131] In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by a percentage in the range of at or about 1% to 10%, 10% to 20%, 20% to 30%, 30% to 40%, 40% to 50%, 50% to 60%, 60% to 70%, 70% to 80%, 80% to 90%, or 90% to 100%.

[00132] In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by at least at or about 1, 2, 3, 4, 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, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99%.

[00133] In some embodiments, the rapid second expansion is performed after the activation of T cells effected by the priming first expansion has decreased by up to at or about 1, 2, 3, 4, 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, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%.

[00134] In some embodiments, the decrease in the activation of T cells effected by the priming first expansion is determined by a reduction in the amount of interferon gamma released by the T cells in response to stimulation with antigen.

[00135] In some embodiments, the priming first expansion of T cells is performed during a period of up to at or about 7 days or about 8 days.

[00136] In some embodiments, the priming first expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days.

[00137] In some embodiments, the priming first expansion of T cells is performed during a period of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days.

[00138] In some embodiments, the rapid second expansion of T cells is performed during a period of up to at or about 11 days.

[00139] In some embodiments, the rapid second expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

[00140] In some embodiments, the rapid second expansion of T cells is performed during a period of 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

[00141] In some embodiments, the priming first expansion of T cells is performed during a period of from at or about 1 day to at or about 7 days and the rapid second expansion of T cells is performed during a period of from at or about 1 day to at or about 11 days.

[00142] In some embodiments, the priming first expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days and the rapid second expansion of T cells is performed during a period of up to at or about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days or 11 days.

[00143] In some embodiments, the priming first expansion of T cells is performed during a period of from at or about 1 day to at or about 8 days and the rapid second expansion of T cells is performed during a period of from at or about 1 day to at or about 9 days.

[00144] In some embodiments, the priming first expansion of T cells is performed during a period of 8 days and the rapid second expansion of T cells is performed during a period of 9 days.

[00145] In some embodiments, the priming first expansion of T cells is performed during a period of from at or about 1 day to at or about 7 days and the rapid second expansion of T cells is performed during a period of from at or about 1 day to at or about 9 days.

[00146] In some embodiments, the priming first expansion of T cells is performed during a period of 7 days and the rapid second expansion of T cells is performed during a period of 9 days.

[00147] In some embodiments, the T cells are tumor infiltrating lymphocytes (TILs).

[00148] In some embodiments, the T cells are marrow infiltrating lymphocytes (MILs).

[00149] In some embodiments, the T cells are peripheral blood lymphocytes (PBLs).

[00150] In some embodiments, the T cells are obtained from a donor suffering from a cancer.

[00151] In some embodiments, the T cells are TILs obtained from a tumor excised from a patient suffering from a cancer.

[00152] In some embodiments, the T cells are MILs obtained from bone marrow of a patient suffering from a hematologic malignancy.

[00153] In some embodiments, the T cells are PBLs obtained from peripheral blood mononuclear cells (PBMCs) from a donor. In some embodiments, the donor is suffering from a cancer. In some embodiments, the cancer is the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, endometrial cancer, thyroid cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)),

glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the donor is suffering from a tumor. In some embodiments, the tumor is a liquid tumor. In some embodiments, the tumor is a solid tumor. In some embodiments, the donor is suffering from a hematologic malignancy.

[00154] In certain aspects of the present disclosure, immune effector cells, *e.g.*, T cells, can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as FICOLL separation. In one preferred aspect, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one aspect, the cells collected by apheresis may be washed to remove the plasma fraction and, optionally, to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. In one aspect, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL gradient or by counterflow centrifugal elutriation.

[00155] In some embodiments, the T cells are PBLs separated from whole blood or apheresis product enriched for lymphocytes from a donor. In some embodiments, the donor is suffering from a cancer. In some embodiments, the cancer is the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, endometrial cancer, thyroid cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM),

gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the donor is suffering from a tumor. In some embodiments, the tumor is a liquid tumor. In some embodiments, the tumor is a solid tumor. In some embodiments, the donor is suffering from a hematologic malignancy. In some embodiments, the PBLs are isolated from whole blood or apheresis product enriched for lymphocytes by using positive or negative selection methods, i.e., removing the PBLs using a marker(s), *e.g.*, CD3+ CD45+, for T cell phenotype, or removing non-T cell phenotype cells, leaving PBLs. In other embodiments, the PBLs are isolated by gradient centrifugation. Upon isolation of PBLs from donor tissue, the priming first expansion of PBLs can be initiated by seeding a suitable number of isolated PBLs (in some embodiments, approximately 1×10^7 PBLs) in the priming first expansion culture according to the priming first expansion step of any of the methods described herein.

[00156] An exemplary TIL process known as process 3 (also referred to herein as Gen 3) containing some of these features is depicted in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C and/or Figure 8D), and in Figure 36, and some of the advantages of this embodiment of the present invention over Gen 2 are described in Figures 1, 2, 8, 30, and 31 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P). Embodiments of Gen 3 are shown in Figures 1, 8, 30, and 36 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P). Process 2A or Gen 2 or Gen 2A is also described in U.S. Patent Publication No. 2018/0280436, incorporated by reference herein in its entirety. The Gen 3 process is also described in International Patent Publication WO 2020/096988.

[00157] As discussed and generally outlined herein, TILs are taken from a patient sample and manipulated to expand their number prior to transplant into a patient using the TIL expansion process described herein and referred to as Gen 3. In some embodiments, the TILs may be optionally genetically manipulated as discussed below. In some embodiments, the TILs may be cryopreserved prior to or after expansion. Once thawed, they may also be restimulated to increase their metabolism prior to infusion into a patient.

[00158] In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G

and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step B) is shortened to 1 to 8 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step D) is shortened to 1 to 9 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step B) is shortened to 1 to 8 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step D) is shortened to 1 to 8 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step B) is shortened to 1 to 7 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step D) is shortened to 1 to 9 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (including processes referred herein as the pre-Rapid Expansion (Pre-REP), as well as processes shown in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C) as Step B) is 1 to 7 days and the rapid second expansion (including processes referred to herein as Rapid Expansion Protocol (REP) as well as processes shown in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or

Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) as Step D) is 1 to 10 days, as discussed in detail below as well as in the examples and figures. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) is shortened to 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 7 to 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 8 to 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is shortened to 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 7 to 8 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is shortened to 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or

Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 8 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 7 to 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G

and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 8 to 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)) is 9 to 10 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) is shortened to 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) is 7 to 9 days. In some embodiments, the combination of the priming first expansion and rapid second expansion (for example, expansions described as Step B and Step D in Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C) is 14-16 days, as discussed in detail below and in the examples and figures. Particularly, it is considered that certain embodiments of the present invention comprise a priming first expansion step in which TILs are activated by exposure to an anti-CD3 antibody, *e.g.*, OKT-3 in the presence of IL-2 or exposure to an antigen in the presence of at least IL-2 and an anti-CD3 antibody *e.g.* OKT-3. In certain embodiments, the TILs which are activated in the priming first expansion step as described above are a first population of TILs *i.e.*, which are a primary cell population.

[00159] The "Step" Designations A, B, C, *etc.*, below are in reference to the non-limiting example in Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) and in reference to certain non-limiting embodiments described herein. The ordering of the Steps below and in

Figure 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P) 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

[00160] In general, TILs are initially obtained from a patient tumor sample ("primary TILs") or from circulating lymphocytes, such as peripheral blood lymphocytes, including peripheral blood lymphocytes having TIL-like characteristics, and are then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, and optionally evaluated for phenotype and metabolic parameters as an indication of TIL health.

[00161] A patient tumor sample may be obtained using methods known in the art, generally via surgical resection, needle biopsy or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. The solid tumor may be of any cancer type, including, but not limited to, breast, pancreatic, prostate, colorectal, lung, brain, renal, stomach, and skin (including but not limited to squamous cell carcinoma, basal cell carcinoma, and melanoma). In some embodiments, the cancer is selected from cervical cancer, head and neck cancer (including, for example, head and neck squamous cell carcinoma (HNSCC)), glioblastoma (GBM), gastrointestinal cancer, ovarian cancer, sarcoma, pancreatic cancer, bladder cancer, breast cancer, triple negative breast cancer, and non-small cell lung carcinoma. In some embodiments, the cancer is melanoma. In some embodiments, useful TILs are obtained from malignant melanoma tumors, as these have been reported to have particularly high levels of TILs.

[00162] Once obtained, the tumor sample is generally fragmented using sharp dissection into small pieces of between 1 to about 8 mm^3, with from about 2-3 mm^3 being particularly useful. The TILs are cultured from these fragments using enzymatic tumor digests. Such tumor digests may be produced by incubation in enzymatic media (*e.g.*, Roswell Park Memorial Institute (RPMI) 1640 buffer, 2 mM glutamate, 10 mcg/mL 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_2, followed by repeated cycles of mechanical dissociation and incubation under the foregoing conditions until only small tissue pieces are present. At the end of this process, if the cell suspension contains a large number of red blood cells or dead cells, a density gradient separation using FICOLL branched hydrophilic polysaccharide may be performed to remove these cells. Alternative methods known in the art may be used, such as those described in U.S. Patent Application Publication No. 2012/0244133 A1, the disclosure of which is incorporated by reference herein. Any of the foregoing methods may be used in any of the embodiments described herein for methods of expanding TILs or methods treating a cancer.

[00163] 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.

[00164] 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.

[00165] 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.

[00166] 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.

[00167] 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.

[00168] 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.

[00169] In some embodiment, the enzyme mixture includes about 10.2-ul of neutral protease (0.36 DMC U/mL), 21.3 µL of collagenase (1.2 PZ/mL) and 250-ul of DNAse I (200 U/mL) in about 4.7 mL of sterile HBSS.

[00170] 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 in an enzyme mixture comprising collagenase, DNase, and hyaluronidase. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% CO_2. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and hyaluronidase for 1-2 hours at 37°C, 5% CO_2 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_2 with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[00171] In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and neutral protease. In some embodiments, the tumors are digested in in an enzyme mixture comprising collagenase, DNase, and neutral protease for 1-2 hours. 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_2. 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_2 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_2 with constant rotation. In some embodiments, the whole tumor is combined with the enzymes to form a tumor digest reaction mixture.

[00172] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00173] 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.

[00174] 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.

[00175] 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.

[00176] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 1000 IU/mL DNAse, and 1 mg/mL hyaluronidase.

[00177] In some embodiments, the enzyme mixture comprises 10 mg/mL collagenase, 500 IU/mL DNAse, and 1 mg/mL hyaluronidase.

[00178] In general, the cell suspension obtained from the tumor is called a "primary cell population" or a "freshly obtained" or a "freshly isolated" cell population. In certain embodiments, the freshly obtained cell population of TILs is exposed to a cell culture medium comprising antigen presenting cells, IL-12 and OKT-3.

[00179] 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.

[00180] 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 8 (in particular, e.g., Figure 8A and/or Figure 8B and/or Figure 8C and/or Figure 8D and/or Figure 8E and/or Figure 8F and/or Figure 8G and/or Figure 8H and/or Figure 8I and/or Figure 8J and/or Figure 8K and/or Figure 8L and/or Figure 8M and/or Figure 8N and/or Figure 8O and/or Figure 8P)). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the step of fragmentation is an *in vitro* or *ex-vivo* process. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the priming first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the priming first expansion. In some embodiments, the tumor is fragmented and 40 fragments or pieces are placed in each container for the priming first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3. In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 mm^3 to about 1500 mm^3. In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3. In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams. In some embodiments, the multiple fragments comprise about 4 fragments.

[00181] In some embodiments, the TILs are obtained from tumor fragments. In some embodiments, the tumor fragment is obtained by sharp dissection. In some embodiments, the tumor fragment is between about 1 mm^3 and 10 mm^3. In some embodiments, the tumor fragment is between about 1 mm^3 and 8 mm^3. In some embodiments, the tumor fragment is about 1 mm^3. In some embodiments, the tumor fragment is about 2 mm^3. In some embodiments, the tumor fragment is about 3 mm^3. In some embodiments, the tumor fragment is about 4 mm^3. In some embodiments, the tumor fragment is about 5 mm^3. In some embodiments, the tumor fragment is about 6 mm^3. In some embodiments, the tumor fragment is about 7 mm^3. In some embodiments, the tumor fragment is about 8 mm^3. In some embodiments, the tumor fragment is about 9 mm^3. In some embodiments, the tumor fragment is about

10 mm^3. In some embodiments, the tumor fragments are 1-4 mm x 1-4 mm x 1-4 mm. In some embodiments, the tumor fragments are 1 mm x 1 mm x 1 mm. In some embodiments, the tumor fragments are 2 mm x 2 mm x 2 mm. In some embodiments, the tumor fragments are 3 mm x 3 mm x 3 mm. In some embodiments, the tumor fragments are 4 mm x 4 mm x 4 mm.

[00182] In some embodiments, the tumors are fragmented in order to minimize the amount of hemorrhagic, necrotic, and/or fatty tissues on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of hemorrhagic tissue on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of necrotic tissue on each piece. In some embodiments, the tumors are fragmented in order to minimize the amount of fatty tissue on each piece. In certain embodiments, the step of fragmentation of the tumor is an *in vitro* or *ex-vivo* method.

[00183] 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_2 and it then mechanically disrupted again for approximately 1 minute. After being incubated again for 30 minutes at 37 °C in 5% CO_2, the tumor can be mechanically disrupted a third time for approximately 1 minute. In some embodiments, after the third mechanical disruption if large pieces of tissue were present, 1 or 2 additional mechanical dissociations were applied to the sample, with or without 30 additional minutes of incubation at 37 °C in 5% CO_2. In some embodiments, at the end of the final incubation if the cell suspension contained a large number of red blood cells or dead cells, a density gradient separation using Ficoll can be performed to remove these cells.

[00184] In some embodiments, the cell suspension prior to the priming first expansion step is called a "primary cell population" or a "freshly obtained" or "freshly isolated" cell population.

[00185] In some embodiments, cells can be optionally frozen after sample isolation (*e.g.*, after obtaining the tumor sample and/or after obtaining the cell suspension from the tumor sample) 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 8 (in particular, *e.g.*, Figure 8B).

1. Core/Small Biopsy Derived TILs

[00186] In some embodiments, TILs are initially obtained from a patient tumor sample ("primary TILs") obtained by a core biopsy or similar procedure and then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, and optionally evaluated for phenotype and metabolic parameters.

[00187] In some embodiments, a patient tumor sample may be obtained using methods known in the art, generally via small biopsy, core biopsy, needle biopsy or other means for obtaining a sample that contains a mixture of tumor and TIL cells. In general, the tumor sample may be from any solid tumor, including primary tumors, invasive tumors or metastatic tumors. The tumor sample may also be a liquid tumor, such as a tumor obtained from a hematological malignancy. In some embodiments, the sample can be from multiple small tumor samples or biopsies. In some embodiments, the sample can comprise multiple tumor samples from a single tumor from the same patient. In some embodiments, the sample can comprise multiple tumor samples from one, two, three, or four tumors from the same patient. In some embodiments, the sample can comprise multiple tumor samples from multiple tumors from the same patient. The solid tumor may be a lung and/or non-small cell lung carcinoma (NSCLC).

[00188] In general, the cell suspension obtained from the tumor core or fragment is called a "primary cell population" or a "freshly obtained" or a "freshly isolated" cell population. In certain embodiments, the freshly obtained cell population of TILs is exposed to a cell culture medium comprising antigen presenting cells, IL-2 and OKT-3.

[00189] In some embodiments, if the tumor is metastatic and the primary lesion has been efficiently treated/removed in the past, removal of one of the metastatic lesions may be needed. In some embodiments, the least invasive approach is to remove a skin lesion, or a lymph node on the neck or axillary area when available. In some embodiments, a skin lesion is removed or small biopsy thereof is removed. In some embodiments, a lymph node or small biopsy thereof is removed. In some embodiments, the tumor is a melanoma. In some embodiments, the small biopsy for a melanoma comprises a mole or portion thereof.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 4
CONTENANT LES PAGES 1 À 224

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 4
CONTAINING PAGES 1 TO 224

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED IS:

1. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;
(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;
(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 agonist and anti-CD28 agonist beads or antibodies, for 1-7 days, to produce a third population of TILs;
(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and
(e) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

2. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained and/or received from a tumor tissue resected from a subject or patient in a first cell culture medium comprising IL-2 for about 3-9 days to produce a second population of TILs;
(b) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 agonist and anti-CD28 agonist beads or antibodies, for 1-7 days, to produce a third population of TILs;
(c) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs; and
(d) culturing the fourth population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

3. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;
(b) performing an initial expansion (or priming first expansion) of the first population of TILs

in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 8 days;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 agonist and anti-CD28 agonist beads or antibodies, for 1-6 days, to produce a third population of TILs;
(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;
(e) performing a rapid second expansion of the fourth population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

4. A method of expanding tumor infiltrating lymphocytes into a therapeutic population of TILs, the method comprising the steps of:

(a) obtaining and/or receiving a first population of TILs from a sample of tumor tissue produced by surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining tumor tissue from a patient or subject;
(b) adding the tumor tissue into a closed system and performing a first expansion by culturing the first population of TILs in a first 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 3-9 days to obtain the second population of TILs;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or CD3 agonist and CD28 agonist beads or antibodies, for 1-7 days, to produce a third population of TILs;
(d) gene-editing at least a portion of the third population of TILs, to produce a fourth population of TILs;
(e) performing a second expansion by culturing the fourth population of TILs in a second cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fifth population of TILs, wherein the second expansion is performed for about 5-15 days to

obtain the fifth population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, wherein the fifth population of TILs is a therapeutic population of TILs; and

(f) harvesting the therapeutic population of TILs obtained from step (e), wherein each of steps (b) to (f) is performed in a closed, sterile system, and wherein the transition from step (b) to step (c), the transition from step (c) to step (d), the transition from step (d) to step (e) and/or the transition from step (e) to step (f) occurs without opening the system.

5. The method of any one of claims 1-4, further comprising:

digesting in an enzyme media the tumor tissue to produce a tumor digest.

6. The method of claim 5, wherein the enzymatic media comprises a DNase.

7. The method of claim 5 or 6, wherein the enzymatic media comprises a collagenase.

8. The method of any one of claims 5-7, wherein the enzymatic media comprises a neutral protease.

9. The method of any one of claims 5-8, wherein the enzymatic media comprises a hyaluronidase.

10. The method of any of claims 1-9, wherein the step of culturing or rapid second expansion of the fourth population of TILs is performed by culturing the fourth population of TILs in the second cell culture medium for a first period of about 1-7 days, at the end of the first period the fourth population of TILs is split into a plurality of subcultures, each of the subcultures is cultured in a third cell culture medium comprising IL-2 for a second period of about 3-7 days, and at the end of the second period the subcultures are combined to provide the expanded number of TILs or the therapeutic population of TILs.

11. The method of claim 10, wherein the first period of culturing is about 5 days.

12. The method of claim 10 or 11, wherein the second period of culturing is about 4 days.

13. The method of claim 10 or 11, wherein the second period of culturing is about 5 days.

14. The method of any one of claims 1-13, wherein the step of activating the second population of TILs is performed using anti-CD3 agonist beads or antibodies.

15. The method of claim 14, wherein the step of activating the second population of TILs is performed using OKT-3.

16. The method of claim 15, wherein the step of activating the second population of TILs is performed using OKT-3 at 300 ng/mL.

17. The method of any one of claims 1-16, wherein the step of activating the second population of TILs is performed using anti-CD3 agonist and anti-CD28 agonist beads or antibodies.

18. The method of claim 17, wherein the step of activating the second population of TILs is performed using TransAct.

19. The method of claim 18, wherein the step of activating the second population of TILs is performed using TransAct at 1:10, 1:17.5 or 1:100 dilution.

20. The method of any one of claims 1-19, wherein the step of activating the second population of TILs is performed for about 2 days.

21. The method of any one of claims 1-19, wherein the step of activating the second population of TILs is performed for about 3 days.

22. The method of any one of claims 1-19, wherein the step of activating the second population of TILs is performed for about 4 days.

23. The method of any one of claims 1-19, wherein the step of activating the second population of TILs is performed for about 5 days.

24. The method of any one of claims 1-23, wherein the step of culturing the first population of TILs is performed for about 3 days.

25. The method of any one of claims 1-23, wherein the step of culturing the first population of TILs is performed for about 5 days.

26. The method of any one of claims 1-23, wherein the step of culturing the first population of TILs is performed for about 7 days.

27. The method of any one of claims 1-26, wherein the step of culturing the fourth population of TILs is performed for about 8 days.

28. The method of any one of claims 1-26, wherein the step of culturing the fourth population of TILs is performed for about 9 days.

29. The method of any one of claims 1-26, wherein the step of culturing the fourth population of TILs is performed for about 8-9 days.

30. The method of any one of claims 1-26, wherein the step of culturing the fourth population of TILs is performed for about 10 days.

31. The method of any one of claims 1-26, wherein the step of culturing the fourth population of TILs is performed for about 8-10 days.

32. The method of any of claims 1-31, wherein all steps are completed within a period of about 22 days.

33. The method of any of claims 1-31, wherein all steps are completed within a period of about 19-22 days.

34. The method of any of claims 1-31, wherein all steps are completed within a period of about 19-20 days.

35. The method of any of claims 1-31, wherein all steps are completed within a period of about 20-22 days.

36. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;
(b) culturing the first population of TILs in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;
(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and
(d) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

37. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained and/or received from a tumor tissue resected from a subject or patient in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;
(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and
(c) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

38. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor tissue resected from a subject or patient;
(b) performing an initial expansion (or priming first expansion) of the first population of TILs in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 8 days;

(c) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and
(d) performing a rapid second expansion of the third population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

39. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) performing an initial expansion (or priming first expansion) of a first population of TILs obtained and/or received from a tumor tissue resected from a subject or patient in a first cell culture medium to obtain a second population of TILs, wherein the first cell culture medium comprises IL-2, optionally OKT-3, and optionally antigen presenting cells (APCs), wherein the priming first expansion occurs for a period of about 3 to 8 days;

(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and

(c) performing a rapid second expansion of the third population of TILs in a second cell culture medium to obtain an expanded number of TILs, wherein the second cell culture medium comprises IL-2, OKT-3, and APCs; and wherein the rapid expansion is performed over a period of 14 days or less, optionally the rapid second expansion can proceed for about 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion.

40. The method of any one of claims 36-39, further comprising:

digesting in an enzyme media the tumor tissue to produce a tumor digest.

41. The method of claim 40, wherein the enzymatic media comprises a DNase.

42. The method of claim 40 or 41, wherein the enzymatic media comprises a collagenase.

43. The method of any one of claims 40-42, wherein the enzymatic media comprises a neutral protease.

44. The method of any one of claims 40-43, wherein the enzymatic media comprises a hyaluronidase.

45. A method for preparing expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) culturing a first population of TILs obtained by digesting in an enzyme media a tumor tissue resected from a subject or patient to produce a tumor digest in a first cell culture medium comprising IL-2 and OKT-3 for about 3-9 days to produce a second population of TILs;
(b) gene-editing at least a portion of the second population of TILs, to produce a third population of TILs; and
(c) culturing the third population of TILs in a second cell culture medium comprising antigen presenting cells (APCs), OKT-3, and IL-2 for about 5-15 days, to produce an expanded number of TILs.

46. The method of any one of claims 36-45, wherein the step of culturing or initial expansion of the first population of TILs comprises culturing the first population of TILs in the first cell culture medium comprising IL-2 for about 3 days followed by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3 for 2-6 days.

47. The method of any of claims 36-46, wherein the step of culturing or rapid second expansion of the third population of TILs is performed by culturing the third population of TILs in the second cell culture medium for a first period of about 1-7 days, at the end of the first period the third population of TILs is split into a plurality of subcultures, each of the subcultures is cultured in a third cell culture medium comprising IL-2 for a second period of about 3-7 days, and at the end of the second period the subcultures are combined to provide the expanded number of TILs.

48. The method of claim 47, wherein the first period of culturing is about 5 days.

49. The method of claim 47 or 48, wherein the second period of culturing is about 4 days.

50. The method of claim 47 or 48, wherein the second period of culturing is about 5 days.

51. The method of any one of claims 36-50, wherein the step of culturing the first population of TILs is performed for about 3 days.

52. The method of any one of claims 36-50, wherein the step of culturing the first population of TILs is performed for about 5 days.

53. The method of any one of claims 36-50, wherein the step of culturing the first population of TILs is performed for about 7 days.

54. The method of any one of claims 36-53, wherein the step of culturing the third population of TILs is performed for about 8 days.

55. The method of any one of claims 36-53, wherein the step of culturing the third population of TILs is performed for about 9 days.

56. The method of any one of claims 36-53, wherein the step of culturing the third population of TILs is performed for about 8-9 days.

57. The method of any one of claims 36-53, wherein the step of culturing the third population of TILs is performed for about 10 days.

58. The method of any one of claims 36-53, wherein the step of culturing the third population of TILs is performed for about 8-10 days.

59. The method of any one of claims 36-58, wherein all steps are completed within a period of about 22 days.

60. The method of any one of claims 36-58, wherein all steps are completed within a period of about 20 days.

61. The method of any one of claims 36-58, wherein all steps are completed within a period of about 22 days.

62. The method of any one of claims 36-58, wherein all steps are completed within a period of about 19-22 days.

63. The method of any one of claims 36-58, wherein all steps are completed within a period of about 19-20 days.

64. The method of any one of claims 36-58, wherein all steps are completed within a period of about 20-22 days.

65. The method of any one of claims 36-58, wherein all steps are completed within a period of about 16-18 days.

66. The method of any one of claims 36-65, wherein in the step of culturing or initial expansion of the first population of TILs in the first culture medium further comprises anti-CD3 and anti-CD28 beads or antibodies.

67. The method of claim 66, wherein the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct.

68. The method of claim 67, wherein the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct at 1:10, 1:17.5 or 1:100 dilution.

69. The method of any of claims 36-68, wherein the first culture medium comprises OKT-3 at 300 ng/mL.

70. The method of any one of claims 36-69, wherein the step of culturing or initial expansion of the first population of TILs comprises culturing the first population of TILs in the first cell culture medium comprising IL-2 and anti-CD3 and anti-CD28 beads or antibodies for about 3 days followed by culturing the first population of TILs in a cell culture medium comprising IL-2 and OKT-3 for 2-4 days.

71. The method of claim 70, wherein the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct.

72. The method of claim 70, wherein the anti-CD3 and anti-CD28 beads or antibodies comprise TransAct at 1:10, 1:17.5 or 1:100 dilution.

73. The method of any of claims 70-72, wherein the first culture medium comprises OKT-3 at 300 ng/mL.

74. The method of any one of claims 1-3 or 5-68, wherein the expanded number of TILs comprises a therapeutic population of TILs.

75. The method of any one of claims 1-74, wherein the step of gene-editing at least a portion of the second or third population of TILs comprises performing a sterile electroporation step on the second or third population of TILs, wherein the sterile electroporation step mediates the transfer of at least one gene editor.

76. The method of any one of claims 1-74, wherein the step of gene-editing at least a portion of the second or third population of TILs comprises performing a sterile electroporation step on the second or third population of TILs, wherein the sterile electroporation step mediates the transfer of at least two gene editors.

77. The method of claim 76, wherein the electroporation step consists of a single electroporation event that mediates the transfer of the at least two gene editors.

78. The method of claim 76, wherein in the electroporation step for each of the at least two gene editors is transferred individually by an electroporation event independently of the transfer of any other gene editor.

79. The method of claim 78, wherein the electroporation step further comprises a rest period after each electroporation event.

80. The method of claim 79, wherein the electroporation step comprises a first electroporation event that mediates the transfer of a first gene editor for modulating expression of a first protein, a first rest period, a second electroporation event that mediates the transfer of a second gene editor for modulating expression of a second protein, and a second rest period, wherein the first and second rest periods are the same or different.

81. The method of claim 80, wherein the first and second rest periods comprise incubating the third or fourth population of TILs in the second cell culture medium comprising IL-2 and/or IL-15.

82. The method of claim 81, wherein the first and second rest periods comprise incubating the third or fourth population of TILs in the second cell culture medium comprising IL-2 at 300 IU/mL, 1000 IU/mL or 6000 IU/mL.

83. The method of claim 81, wherein the first and second rest periods comprise incubating the third or fourth population of TILs in the second cell culture medium comprising IL-15 at 15 ng/mL.

84. The method of any one of claims 80-78, wherein the first and second rest periods comprise incubating the third or fourth population of TILs at about 30-40 °C with about 5% CO_2.

85. The method of claim 84, wherein the first and second rest periods comprise incubating the third or fourth population of TILs at about 25, 28, 30, 32, 35 or 37 °C with about 5% CO_2.

86. The method of any one of claims 80-85, wherein the first and second rest periods are independently about 10 hours to 5 days.

87. The method of claim 86, wherein the first and second rest periods are independently about 10 hours to 3 days.

88. The method of claim 87, wherein the first rest period is about 1 to 3 days.

89. The method of claim 87, wherein the first rest period is about 3 days.

90. The method of any one of claims 87-89, wherein the second rest period is about 10 hours to 1 day.

91. The method of any one of claims 87-89, wherein the second rest period is about 12 hours to 24 hours.

92. The method of any one of claims 87-89, wherein the second rest period is about 15 hours to about 18 hours.

93. The method of any one of claims 87-89, wherein the second rest period comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about 15 hours to 23 hours at about 30°C.

94. The method of any one of claims 87-89, wherein the second rest period comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about for about one hour at 37°C followed by about 15 hours to 23 hours at about 30°C.

95. The method of any one of claims 87-89, wherein the second rest period comprises incubating the third or fourth population of TILs in a cell culture medium comprising IL-2 for about one hour at 37°C followed by about 15 hours to 22 hours at about 30°C.

96. The method of claim 87, wherein the first rest period is about 3 days and the second rest period is about 10 to 16 hours.

97. The method of any one of claims 75-96, wherein the electroporation step is preceded by washing the second or third population of TILs in a cytoporation buffer.

98. The method of claim 75 or 97, wherein the at least one gene editor is a TALE nuclease system for modulating the expression of at least one protein.

99. The method of claim 98, wherein the at least one gene editor comprises a TALE nuclease system that modulates expression of PD-1.

100. The method of claim 98, wherein the at least one gene editor comprises a TALE nuclease system that modulates expression of CTLA-4.

101. The method of claim 98, wherein the at least one gene editor comprises a TALE nuclease system that modulates expression of LAG-3.

102. The method of claim 98, wherein the at least one gene editor comprises a TALE nuclease system that modulates expression of CISH.

103. The method of claim 98, wherein the at least one gene editor comprises a TALE nuclease system that modulates expression of CBL-B.

104. The method of claim 98, wherein the at least one gene editor comprises a TALE nuclease system that modulates expression of TIGIT.

105. The method of any one of claims 76-97, wherein the at least two gene editors comprise a first gene editor comprising a first TALE nuclease system for modulating expression of a first protein and a second gene editor comprising a second TALE nuclease system for modulating expression of a second protein.

106. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of PD-1, CTLA-4, LAG-3, CISH, TIGIT and/or CBL-B.

107. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of PD-1 and CTLA-4.

108. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of PD-1 and LAG-3.

109. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of PD-1 and CISH.

110. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of PD-1 and CBL-B.

111. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of PD-1 and TIGIT.

112. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of CTLA-4 and LAG-3.

113. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of CTLA-4 and CISH.

114. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of CTLA-4 and CBL-B.

115. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of LAG-3 and CISH.

116. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of LAG-3 and CBL-B.

117. The method of claim 105, wherein the first and second TALE nuclease systems modulate expression of CISH and CBL-B.

118. The method of claim 105, wherein the first protein and the second protein are independently selected from the group consisting of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, with the proviso that the first protein and the second protein are different.

119. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of PD-1 and CTLA-4.

120. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of PD-1 and LAG-3.

121. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of PD-1 and CISH.

122. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of PD-1 and CBL-B.

123. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of PD-1 and TIGIT.

124. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of CTLA-4 and LAG-3.

125. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of CTLA-4 and CISH.

126. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of CTLA-4 and CBL-B.

127. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of LAG-3 and CISH.

128. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of LAG-3 and CBL-B.

129. The method of claim 118, wherein the first protein and the second protein are selected from the group consisting of CISH and CBL-B.

130. The method of claim 118, wherein the first protein is PD-1 and the second protein is CTLA-4.

131. The method of claim 118, wherein the first protein is CTLA-4 and the second protein is PD-1.

132. The method of claim 118, wherein the first protein is PD-1 and the second protein is LAG-3.

133. The method of claim 118, wherein the first protein is LAG-3 and the second protein is PD-1.

134. The method of claim 118, wherein the first protein is PD-1 and the second protein is CISH.

135. The method of claim 118, wherein the first protein is CISH and the second protein is PD-1.

136. The method of claim 118, wherein the first protein is PD-1 and the second protein is CBL-B.

137. The method of claim 118, wherein the first protein is CBL-B and the second protein is PD-1.

138. The method of claim 118, wherein the first protein is PD-1 and the second protein is TIGIT.

139. The method of claim 118, wherein the first protein is TIGIT and the second protein is PD-1.

140. The method of claim 118, wherein the first protein is CTLA-4 and the second protein is LAG-3.

141. The method of claim 118, wherein the first protein is LAG-3 and the second protein is CTLA-4.

142. The method of claim 118, wherein the first protein is CTLA-4 and the second protein is CISH.

143. The method of claim 118, wherein the first protein is CISH and the second protein is CTLA-4.

144. The method of claim 118, wherein the first protein is CTLA-4 and the second protein is CBL-B.

145. The method of claim 118, wherein the first protein is CBL-B and the second protein is CTLA-4.

146. The method of claim 118, wherein the first protein is LAG-3 and the second protein is CISH.

147. The method of claim 118, wherein the first protein is CISH and the second protein is LAG-3.

148. The method of claim 118, wherein the first protein is LAG-3 and the second protein is CBL-B.

149. The method of claim 118, wherein the first protein is CBL-B and the second protein is LAG-3.

150. The method of claim 118, wherein the first protein is CISH and the second protein is CBL-B.

151. The method of claim 118, wherein the first protein is CBL-B and the second protein is CISH.

152. The method of claim 118, wherein the first protein or the second protein is PD-1.

153. The method of claim 118, wherein the first protein or the second protein is CTLA-4.

154. The method of claim 118, wherein the first protein or the second protein is LAG-3.

155. The method of claim 118, wherein the first protein or the second protein is CISH.

156. The method of claim 118, wherein the first protein or the second protein is CBL-B.

157. The method of claim 118, wherein the first protein or the second protein is TIGIT.

158. The method of any of claims 76-97 or 105-157, wherein the first gene editor downregulates expression of the first protein and the second gene editor downregulates expression of the second protein.

159. The method of any one of claims 1-158, wherein the antigen presenting cells (APCs) are PBMCs.

160. The method of claim 159, wherein the PBMCs are irradiated and allogeneic.

161. The method according to any one of claims 1-158, wherein the antigen-presenting cells are artificial antigen-presenting cells.

162. The method according to claim any one of claims 1-161, wherein the IL-2 concentration is about 10,000 IU/mL to about 5,000 IU/mL.

163. The method according to any one of claims 1-162, wherein the first cell culture medium and/or the second cell culture medium further comprises a 4-1BB agonist and/or an OX40 agonist.

164. The method according to any one of claims 1-163, wherein the tumor tissue is processed into multiple tumor fragments.

165. The method according to claim 164, wherein the tumor fragments are added into the closed system.

166. The method according to claim 165, wherein 150 or fewer of the fragments, 100 or fewer of the fragments, or 50 or fewer of the fragments are added into the closed system.

167. A gene-edited population of tumor infiltrating lymphocytes (TILs) comprising an expanded population of TILs wherein the expression of at least one protein is modulated by a gene editor transferred into at least a portion of the expanded population of TILs.

168. The gene-edited population of TILs of claim 167, wherein the gene editor is a TALE nuclease system for modulating the expression of the at least one protein.

169. The gene-edited population of TILs of claim 168, wherein the at least one protein is PD-1.

170. The gene-edited population of TILs of claim 168, wherein the at least one protein is CTLA-4.

171. The gene-edited population of TILs of claim 168, wherein the at least one protein is LAG-3.

172. The gene-edited population of TILs of claim 168, wherein the at least one protein is CISH.

173. The gene-edited population of TILs of claim 168, wherein the at least one protein is CBL-B.

174. The gene-edited population of TILs of claim 168, wherein the at least one protein is TIGIT.

175. The gene-edited population of TILs of claim 167, wherein the expression of at least two proteins is modulated by at least two gene editors transferred into at least a portion of the expanded population of TILs, wherein the at least two gene editors comprise a first gene editor comprising a first TALE nuclease system for modulating expression of a first protein and a second gene editor comprising a second TALE nuclease system for modulating expression of a second protein.

176. The gene-edited population of TILs of claim 175, wherein the first and second proteins are independently selected from the group consisting of PD-1, CTLA-4, LAG-3, CISH, TIGIT and CBL-B, with the proviso that the first protein and the second protein are different.

177. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of PD-1 and CTLA-4.

178. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of PD-1 and LAG-3.

179. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of PD-1 and CISH.

180. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of PD-1 and CBL-B.

181. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of PD-1 and TIGIT.

182. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of CTLA-4 and LAG-3.

183. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of CTLA-4 and CISH.

184. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of CTLA-4 and CBL-B.

185. The gene-edited population of TILs of claim 175, wherein the first and second TALE proteins are selected from the group consisting of LAG-3 and CISH.

186. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of LAG-3 and CBL-B.

187. The gene-edited population of TILs of claim 175, wherein the first and second proteins are selected from the group consisting of CISH and CBL-B.

188. The gene-edited population of TILs of any one of claims 167-187, manufactured by the method of any one of claims 1-166.

189. A pharmaceutical composition comprising the gene edited population of TILs of any one of claims 167-188 and a pharmaceutically acceptable carrier.

190. A method for treating a subject with cancer, the method comprising administering a therapeutically effective dose of the gene edited population of TILs of any one of claims 167-188 or the pharmaceutical composition of claim 189.

191. The method of claim 190, wherein the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer

(NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), renal cancer, and renal cell carcinoma.

192. A method for treating a subject with cancer, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining a first population of TILs from a tumor resected from a patient by processing a tumor sample obtained from the patient into multiple tumor fragments;

(b) adding the tumor fragments into a closed system and performing a first expansion by culturing the first population of TILs in a first 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 3-8 days to obtain the second population of TILs;

(c) activating the second population of TILs using anti-CD3 agonist beads or antibodies, or anti-CD3 and anti-CD28 agonist beads or antibodies, for 1-6 days, to produce a third population of TILs;

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

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

(g) performing a second expansion by culturing the third population of TILs in a second cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs), to produce a fourth population of TILs, wherein the second expansion is performed for about 5-15 days to obtain the third population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, wherein the fourth population of TILs is a therapeutic population of TILs;

(h) harvesting the therapeutic population of TILs obtained from step (e) to provide a harvested TIL population, wherein one or more of steps (a) to (h) are performed in a closed, sterile system;

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

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

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

wherein the electroporation step comprises the delivery of a Transcription Activator-Like Effector Nuclease (TALEN) system for inhibiting the expression of PD-1, CTLA-4, LAG-3, CISH, TIGIT and/or CBL-B.

193. The method of claim 192, wherein the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of PD-1.

194. The method of claim 192, wherein the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of CTLA-4.

195. The method of claim 192, wherein the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of LAG-3.

196. The method of claim 192, wherein the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of CISH.

197. The method of claim 192, wherein the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of CBL-B.

198. The method of claim 192, wherein the electroporation step comprises the delivery of a TALEN system for inhibiting the expression of TIGIT.

199. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and CTLA-4.

200. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and LAG-3.

201. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and CISH.

202. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and CBL-B.

203. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of PD-1 and TIGIT.

204. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and LAG-3.

205. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and CISH.

206. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and CBL-B.

207. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CTLA-4 and TIGIT.

208. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of LAG-3 and CISH.

209. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of LAG-3 and CBL-B.

210. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of LAG-3 and TIGIT.

211. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CISH and CBL-B.

212. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CISH and TIGIT.

213. The method of claim 192, wherein the electroporation step comprises the delivery of TALEN systems for inhibiting the expression of CBL-B and TIGIT.

214. The method of any one of claims 192-213, wherein the therapeutically effective dosage of TILs is from about 1×10^{9} to about 1×10^{11} TILs.

215. The method of any one of claims 192-214, wherein prior to administering a therapeutically effective dosage of the harvested TIL population in step (k), a non-myeloablative lymphodepletion regimen has been administered to the patient.

216. The method of any one of claims 192-215, further comprising the step of treating the patient with a high-dose IL-2 regimen starting on the day after administration of the therapeutically effective dosage of the harvested TIL population to the patient in step (k).

217. The method of any one of claims 192-216, wherein the cancer is selected from the group consisting of melanoma, metastatic melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), metastatic NSCLC, lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), renal cancer, and renal cell carcinoma.

218. The method of any one of claims 192-216, wherein the cancer is melanoma.

219. The method of claim 218, wherein the cancer is metastatic melanoma.

220. The method of any one of claims 192-216, wherein the cancer is NSCLC.

221. The method of claim 220, wherein the cancer is metastatic NSCLC.

222. The method of any one of claims 192-221, wherein the gene-editing causes expression of one or more immune checkpoint genes to be silenced or reduced in at least a portion of the therapeutic population of TILs.

223. Use of a composition, product, process, or system comprising TILs produced by any of the methods described herein, including the use of TILs in the preparation of a medicament for the treatment of a subject with cancer, characterized by one or more elements disclosed in the application.

Process 2A: about 22 days from Steps A - E

1. **STEP A**

Obtain Patient Tumor Sample

2. **STEP B**

Fragmentation and First Expansion

3 days to 14 days

3. **STEP C**

First Expansion to Second Expansion Transition

No Storage and Closed System

4. **STEP D**

Second Expansion

IL-2, OKT-3, and antigen-presenting feeder cells

Closed System

5. **STEP E**

Harvest TILS from Step D

Closed System

6. **STEP E**

Final Formulation and/or Transfer to Infusion Bag

(optionally cryopreserve)

Figure 1

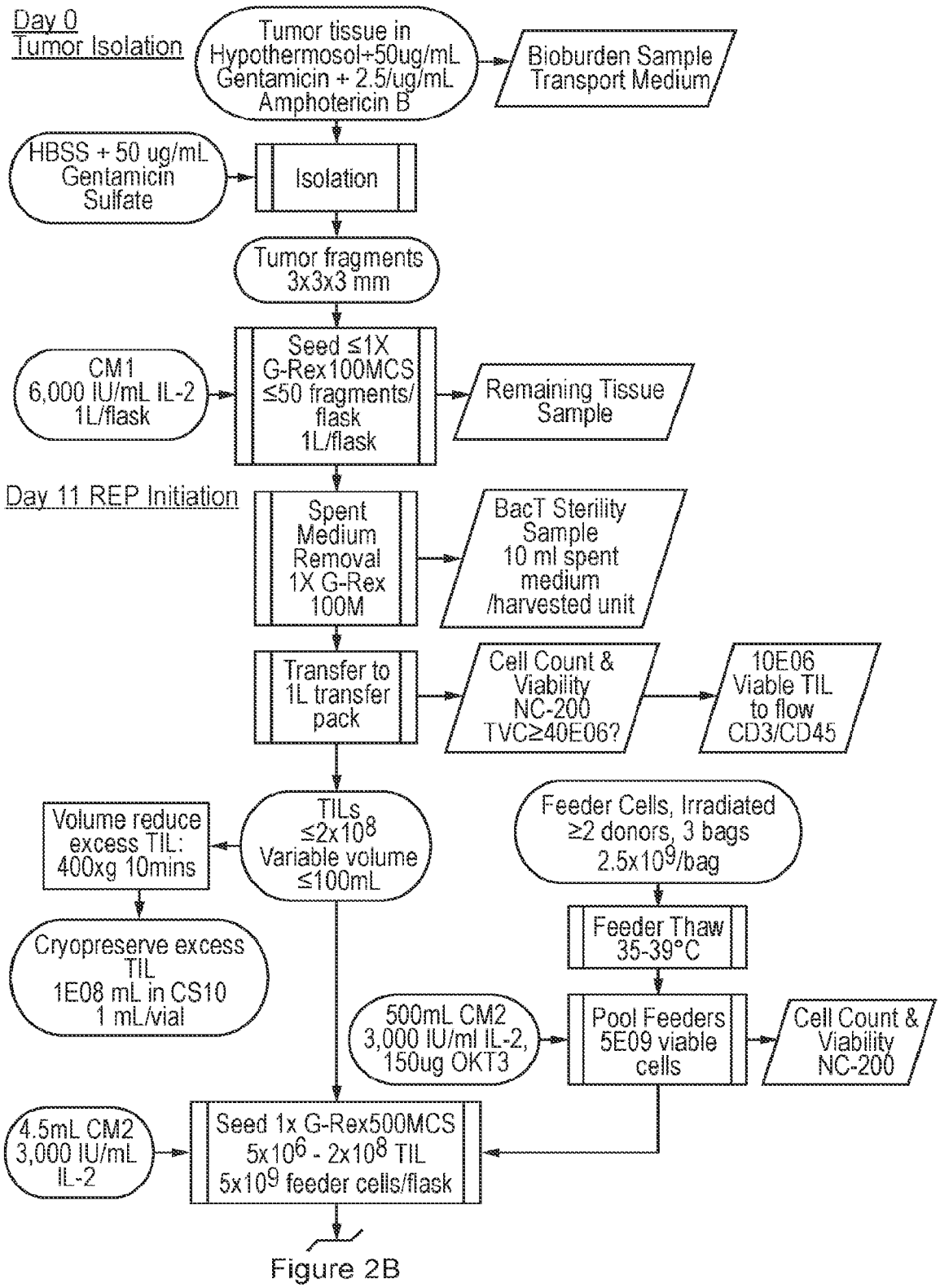

Figure 2B

Figure 2A

Figure 2A

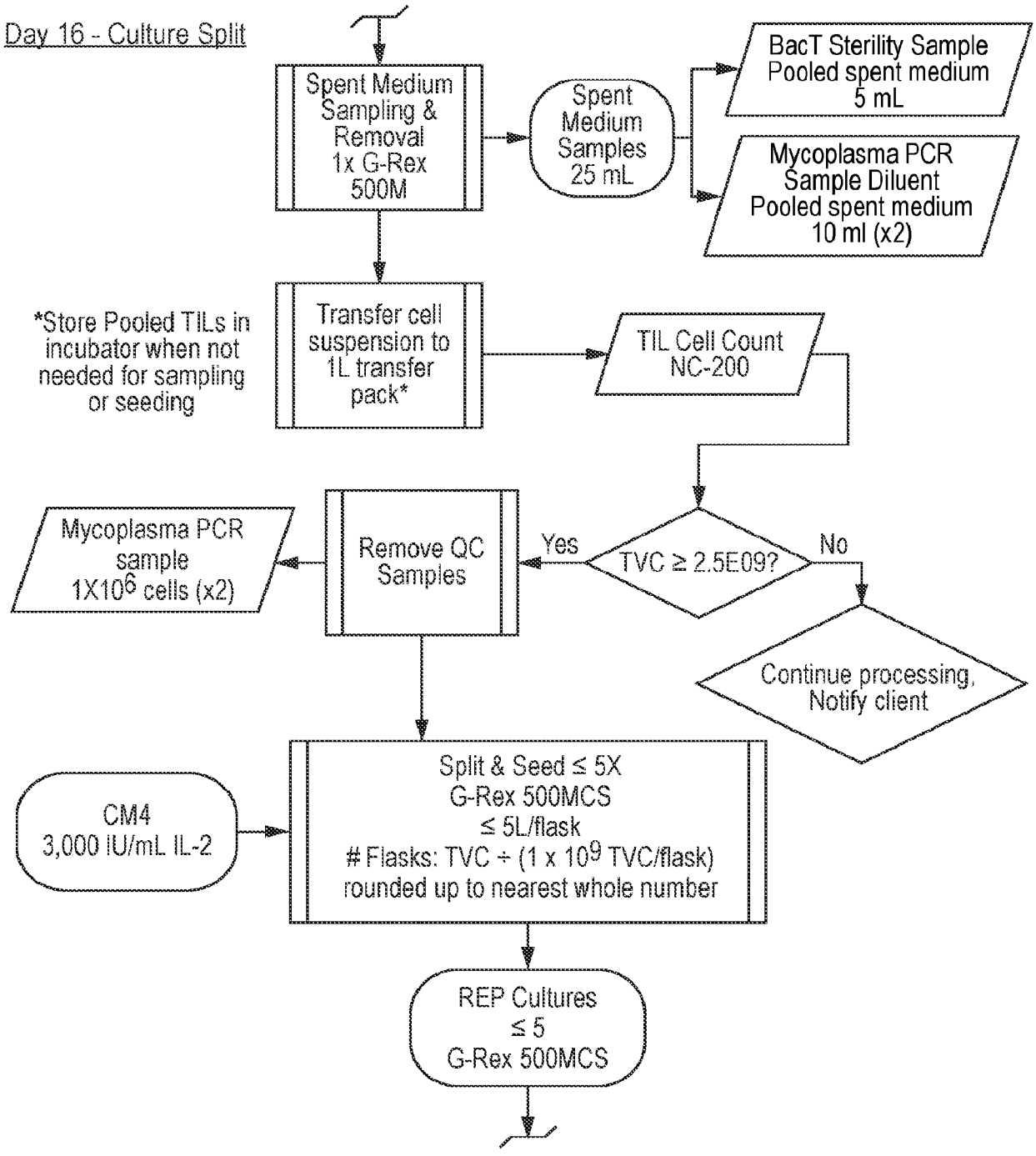

Figure 2C

Figure 2B

Figure 2B

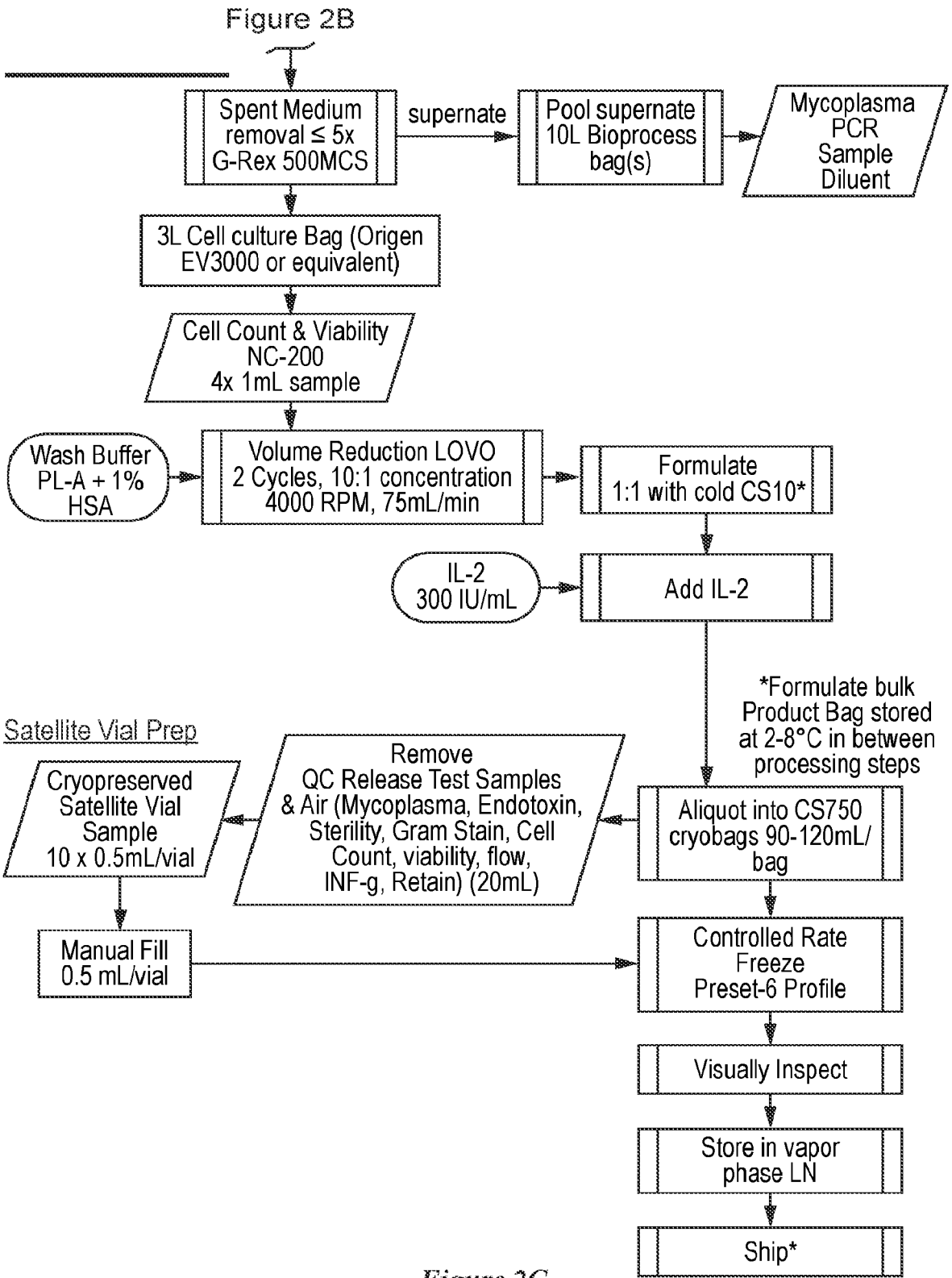

Figure 2C

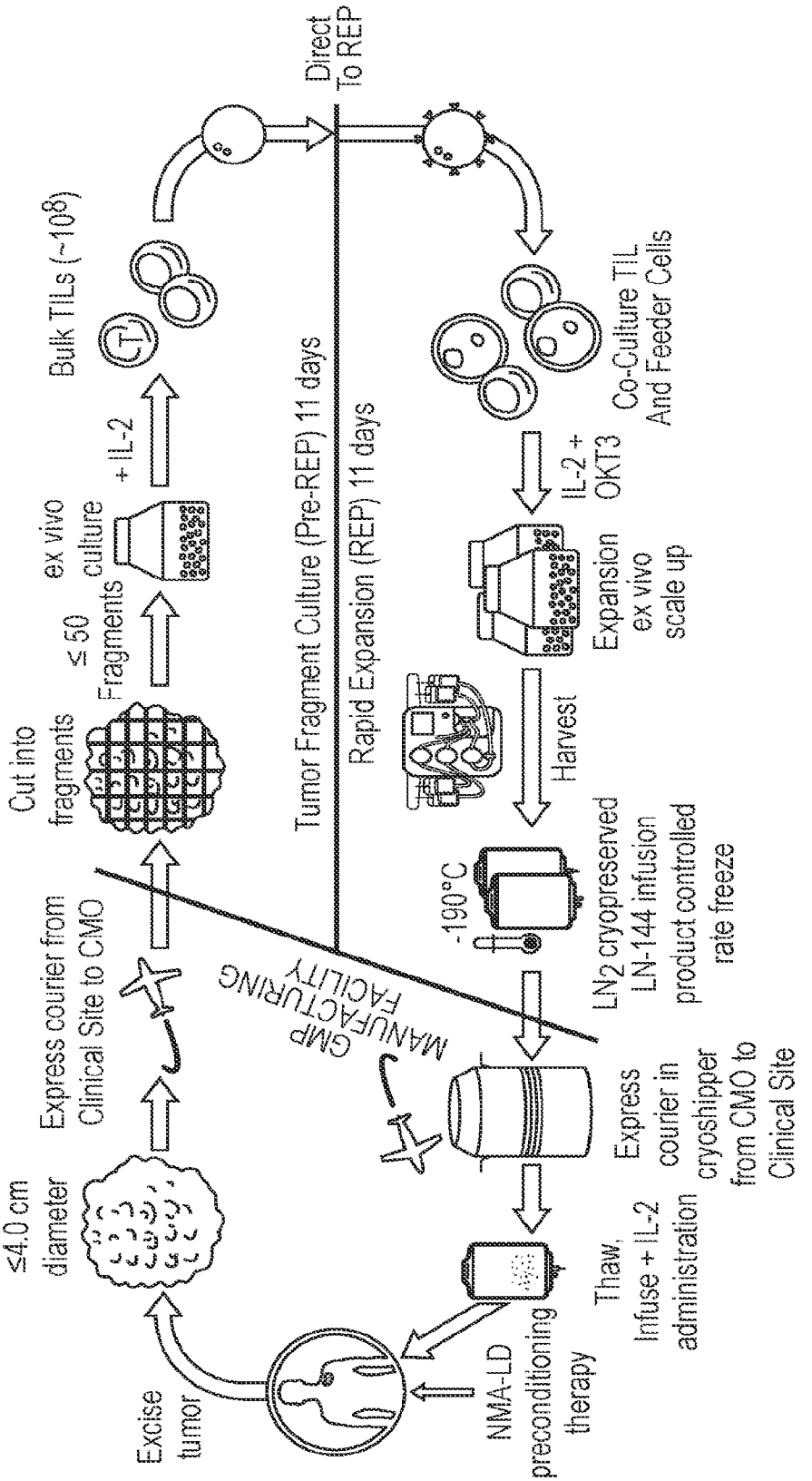
Excise tumor
≤4.0 cm diameter
Express courier from Clinical Site to CMO
NMA-LD preconditioning therapy
Thaw, Infuse + IL-2 administration
Express courier in cryoshipper from CMO to Clinical Site
MANUFACTURING FACILITY
GMP
-190°C
LN2 cryopreserved LN-144 infusion product controlled rate freeze
Harvest
Cut into fragments
≤ 50 Fragments
ex vivo culture
+IL-2
Bulk TILs (~10^8)
Tumor Fragment Culture (Pre-REP) 11 days
Rapid Expansion (REP) 11 days
Direct To REP
Co-Culture TIL And Feeder Cells
IL-2 + OKT3
Expansion ex vivo scale up

Figure 3

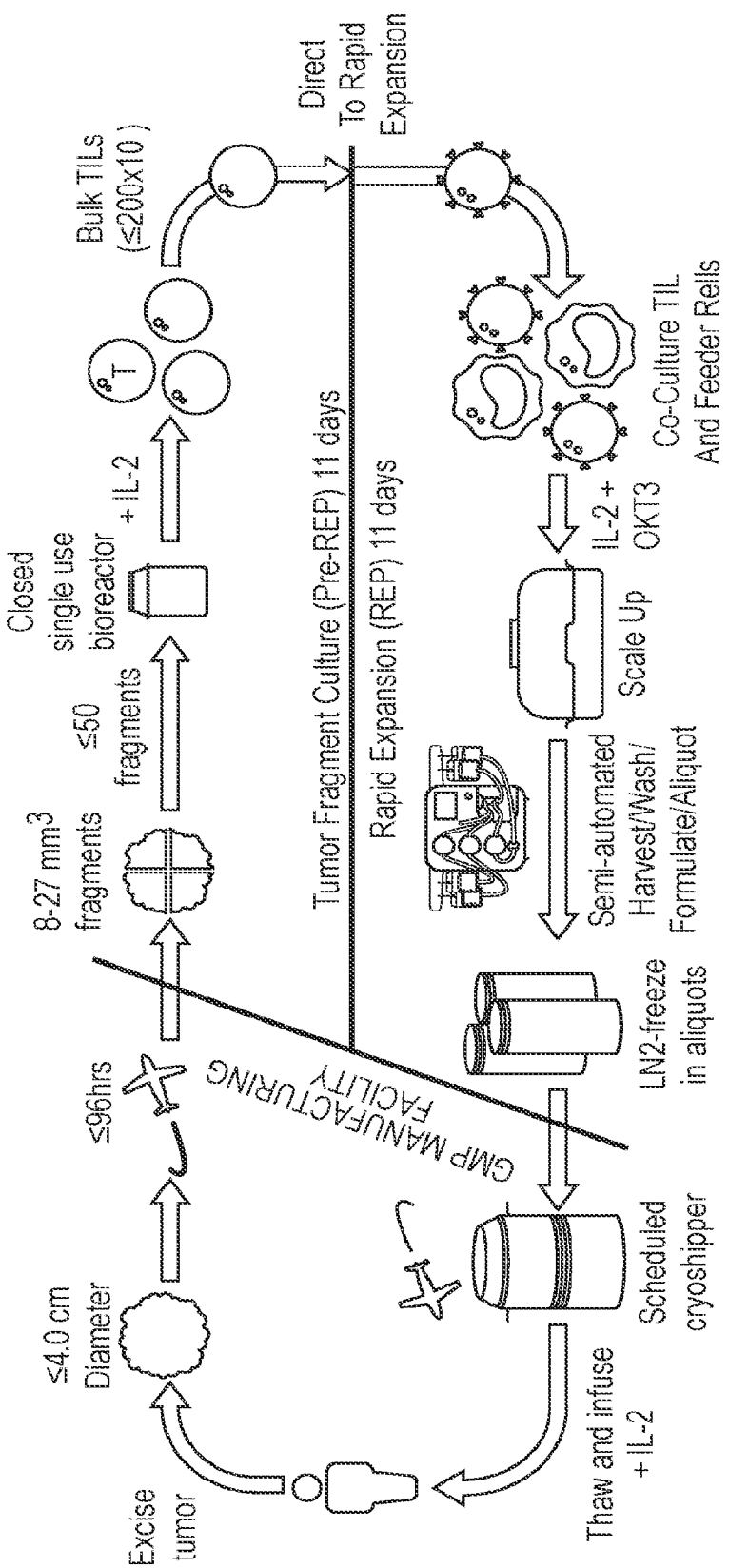
Excise tumor
≤4.0 cm Diameter
≤96hrs
8-27 mm3 fragments
≤50 fragments
Closed single use bioreactor
+ IL-2
Bulk TILs (≤200x10)
Direct To Rapid Expansion
Tumor Fragment Culture (Pre-REP) 11 days
Rapid Expansion (REP) 11 days
Co-Culture TIL And Feeder Rells
IL-2 + OKT3
Scale Up
Semi-automated Harvest/Wash/ Formulate/Aliquot
LN2-freeze in aliquots
Scheduled cryoshipper
Thaw and infuse + IL-2
GMP MANUFACTURING FACILITY

Figure 4

Process 1C: 43-55 Days for Steps A - E	Process 2A: about 22 days from Steps A - E
1. **STEP A** Obtain Patient Tumor Sample	1. **STEP A** Obtain Patient Tumor Sample
2. **STEP B** Fragmentation and First Expansion 11 days to 21 days	2. **STEP B** Fragmentation and First Expansion 3 days to 14 days
3. **STEP C** First Expansion to Second Expansion Transition Optional Storage until Selection	3. **STEP C** First Expansion to Second Expansion Transition No Storage and Closed System
4. **STEP D** Second Expansion IL-2, OKT-3, antigen-presenting feeder cells Optionally repeat one or more times	4. **STEP D** Second Expansion IL-2, OKT-3, antigen-presenting feeder cells Closed System
5. **STEP E** Harvest TILs from Step D	5. **STEP E** Harvest TILs from Step D Closed System
6. **STEP F** Final Formulation and/or Transfer to Infusion Bag	6. **STEP F** Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 5

Process Step	Process 1C Embodiment	Process 2A Embodiment	Advantages
Pre-REP	• 4 fragments per 10 GREX-10 flasks • 11-21 day duration	• 40 fragments per 1 GREX-100M flask • 11 day duration	• Increased tumor fragments per flask • Shortened culture time • Reduced number of steps • Amenable to closed system
Pre-REP to REP Transition	• Pre-REP TIL are frozen until phenotyped for selection then thawed to proceed to the REP (-day 30) • REP requires >40x10^6 TIL	• Pre-REP TIL directly move to REP on day 11 • REP requires 25-200x10^6 TIL	• Shortened pre-REP-to-REP process • Reduced number of steps • Eliminated phenotyping selection • Amenable to closed system
REP	• 6 GREX-100M flasks on REP day 0 • 5x10^6 TIL and 5x10^8 PBMC feeders per flask on REP day 0 • Split to 18-36 flasks on REP day 7 • 14 day duration	• 1 GREX-500M flask on day 11 • 25-200x10^6 TIL and 5x10^9 PBMC feeders on day 11 • Split to ≤ 6 GREX-500M flasks on day 16 • 11 day duration	• Reduced number of steps • Shorter REP duration • Closed system transfer of TIL between flasks • Closed system media exchanges
Harvest	• TIL harvested via centrifugation	• TIL harvested via LOVO automated cell washing system	• Reduced number of steps • Automated cell washing • Closed system • Reduced loss of product during wash
Final Formulation	• Fresh product in Hypothermosol • Single infusion bag • Limited shipping stability	• Cryopreserved product in PlasmaLyte-A + 1% HSA and CS10 stored in LN_2 • Multiple aliquots • Longer shipping stability	• Shipping flexibility • Flexible patient scheduling • More timely release testing
Overall Estimated Process Time	• 43 -55 days	• 22 days	• Faster turnaround to patient

Figure 6

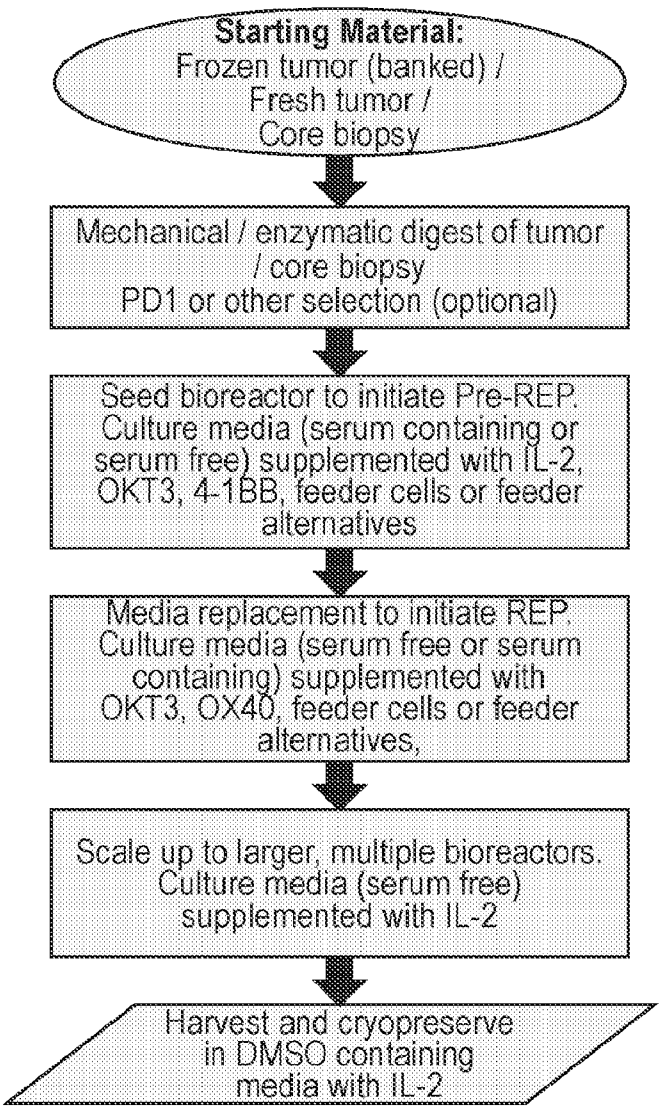
Starting Material:
Frozen tumor (banked) /
Fresh tumor /
Core biopsy
Mechanical / enzymatic digest of tumor / core biopsy
PD1 or other selection (optional)
Seed bioreactor to initiate Pre-REP. Culture media (serum containing or serum free) supplemented with IL-2, OKT3, 4-1BB, feeder cells or feeder alternatives
Media replacement to initiate REP. Culture media (serum free or serum containing) supplemented with OKT3, OX40, feeder cells or feeder alternatives,
Scale up to larger, multiple bioreactors. Culture media (serum free) supplemented with IL-2
Harvest and cryopreserve in DMSO containing media with IL-2
Day 0: Tumor / biopsy prep
Day 0: Pre-REP Initiation
Day 5-8: REP Initiation
Day 9 to 11: REP Scale Up
Day 12 to 16: Harvest

Figure 7

Figure 8A

Process 2A/Gen 2: about 22 days from Steps A-E	Process Gen 3: about 14-18 days from Steps A-E
STEP A Obtain Patient Tumor Sample (optionally can be frozen before Step B)	**STEP A** Obtain Patient Tumor Sample (optionally can be frozen before Step B)
STEP B First Expansion (physical fragmentation to at least 40 fragments per container grown for about 3 days to 14 days with media comprising IL-2)	**STEP B** Priming First Expansion (physical fragmentation of up to 60 fragments per container grown for about 1 days to 7 days with media comprising IL-2, OKT-3, and antigen-presenting feeder cells)
STEP C First Expansion to Second Expansion Transition (Step B TILs directly move to Step D, optionally on Step B day 11)	**STEP C** Priming First Expansion to Rapid Second Expansion Transition (Step B TILs directly move to Step D on day 7)
STEP D Second Expansion (TILs grown in growth media medium comprising IL-2, OKT-3, and antigen-presenting feeder cells in a closed container)	**STEP D** Rapid Second Expansion (TILs grown in growth media medium comprising IL-2, OKT-3, and 2X antigen-presenting feeder cells; Days 10-11 scale up and add additional IL-2)
STEP E Harvest TILS from Step D (TILs harvested via closed system)	**STEP E** Harvest TILS from Step D
STEP F Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)	**STEP F** Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 8B

Process Gen 3: about 14-18 days from Steps A-E

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
Priming First Expansion
(physical fragmentation of up to 60 fragments per container grown for about 1 days to 7 days with media comprising IL-2, OKT-3, and antigen-presenting feeder cells)

STEP C
Priming First Expansion to Rapid Second Expansion Transition
(Step B TILs directly move to Step D on day 7)

STEP D
Rapid Second Expansion
(TILs grown in growth media medium comprising IL-2, OKT-3, and 2X antigen-presenting feeder cells; Days 10-11 scale up and add additional IL-2)

STEP E
Harvest TILS from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8C

Embodiment Gen 3.0: about 14-18 days from Steps A - E	Embodiment Gen 3.1 control: about 14-18 days from Steps A - E	Embodiment Gen 3.1 Test/F: about 14-18 days from Steps A - E
STEP A Obtain Patient Tumor Sample (optionally can be frozen before Step B)	**STEP A** Obtain Patient Tumor Sample (optionally can be frozen before Step B)	**STEP A** Obtain Patient Tumor Sample (optionally can be frozen before Step B)
STEP B Priming First Expansion (physical fragmentation of up to 60 fragments per container grown for about 1 days to 7/8 days with media comprising IL-2)	**STEP B** Priming First Expansion (physical fragmentation of up to 60 fragments per container grown for about 1 days to 7/8 days with media comprising IL-2, and OKT-3)	**STEP B** Priming First Expansion (physical fragmentation of up to 60 fragments per container grown for about 1 days to 7/8 days with media comprising IL-2, OKT-3, and antigen-presenting feeder cells)
STEP C Priming First Expansion to Rapid Second Expansion Transition (Step B TILs directly move to Step D on day 7/8)	**STEP C** Priming First Expansion to Rapid Second Expansion Transition (Step B TILs directly move to Step D on day 7/8)	**STEP C** Priming First Expansion to Rapid Second Expansion Transition (Step B TILs directly move to Step D on day 7/8)
STEP D Rapid Second Expansion (TILs grown in growth media medium comprising IL-2, OKT-3, and antigen-presenting feeder cells; Days 10-11 scale up and add additional IL-2)	**STEP D** Rapid Second Expansion (TILs grown in growth media medium comprising IL-2, OKT-3, and 2X antigen-presenting feeder cells; Days 10-11 scale up and add additional IL-2)	**STEP D** Rapid Second Expansion (TILs grown in growth media medium comprising IL-2, OKT-3, and 2X antigen-presenting feeder cells; Days 10-11 scale up and add additional IL-2)
STEP E Harvest TILS from Step D	**STEP E** Harvest TILS from Step D	**STEP E** Harvest TILS from Step D
STEP F Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)	**STEP F** Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)	**STEP F** Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 8D

Modified Gen 2-like Process: about 22 days from Steps A - E

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B;
optionally tumor sample can be a core/small biopsy)

STEP B1
Initial Culture
physical fragmentation of up to 60 tumor fragments or up to 10 cores/small biopsies per container, TILs grown for 3 days in growth medium comprising IL-2

STEP B2
Priming First Expansion
TILs grown for 8 days in growth medium comprising IL-2, OKT-3, and antigen-presenting feeder cells)

STEP C
Priming First Expansion to Rapid Second Expansion Transition
(Step B TILs directly move to Step D on day 11)

STEP D
Rapid Second Expansion
(volume reduced; TILs grown in growth media medium comprising IL-2, OKT-3, and 50X antigen-presenting feeder cells; Day 16 scale up and add additional IL-2)

STEP E
Harvest TILS from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8E

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing
(culture TILs in medium comprising anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8F

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing
(culture TILs in medium comprising anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8G

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing
(electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8H

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing
(electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8I

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing
(culture TILs in medium comprising OKT3 or anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8J

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing
(culture TILs in medium comprising OKT3 or anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8K

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing
(electroporate TILs with TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8L

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing
(electroporate TILs with TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8M

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing
(culture TILs in medium comprising OKT3 or anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with first set of TALEN mRNAs; rest for about 2 days at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C; electroporate TILs with second set of TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8N

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing
(culture TILs in medium comprising OKT3 or anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with first set of TALEN mRNAs; rest for about 2 days at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C; electroporate TILs with second set of TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8O

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing
(electroporate TILs with first set of TALEN mRNAs; rest for about 2 days at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C; electroporate TILs with second set of TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

Figure 8P

STEP A
Obtain Patient Tumor Sample
(optionally can be frozen before Step B)

STEP B
First Expansion
(physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing
(electroporate TILs with first set of TALEN mRNAs; rest for about 2 days at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C; electroporate TILs with second set of TALEN mRNAs; rest for about 1 day at 25°C, 28°C, 30°C, 32°C, 35°C, or 37°C optionally in media with IL-2 or IL-15)

STEP D
Rapid Second Expansion
(culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag
(optionally cryopreserve)

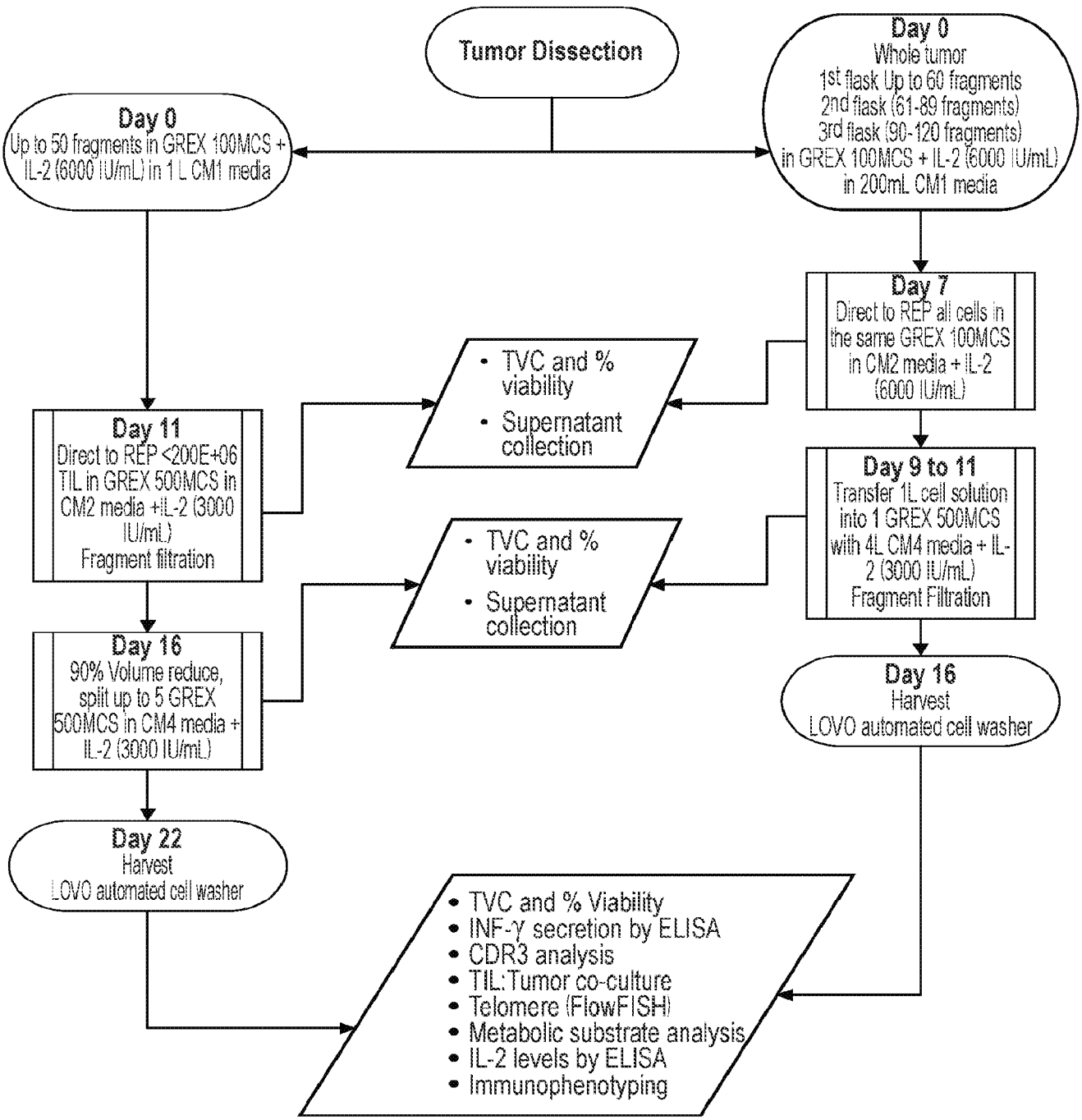
Tumor Dissection
Day 0
Up to 50 fragments in GREX 100MCS + IL-2 (6000 IU/mL) in 1 L CM1 media
Day 0
Whole tumor
1st flask Up to 60 fragments
2nd flask (61-89 fragments)
3rd flask (90-120 fragments)
in GREX 100MCS + IL-2 (6000 IU/mL)
in 200mL CM1 media
Day 7
Direct to REP all cells in the same GREX 100MCS in CM2 media + IL-2 (6000 IU/mL)
• TVC and % viability
• Supernatant collection
Day 11
Direct to REP <200E+06 TIL in GREX 500MCS in CM2 media +IL-2 (3000 IU/mL)
Fragment filtration
Day 9 to 11
Transfer 1L cell solution into 1 GREX 500MCS with 4L CM4 media + IL-2 (3000 IU/mL)
Fragment Filtration
• TVC and % viability
• Supernatant collection
Day 16
90% Volume reduce, split up to 5 GREX 500MCS in CM4 media + IL-2 (3000 IU/mL)
Day 16
Harvest
LOVO automated cell washer
Day 22
Harvest
LOVO automated cell washer
• TVC and % Viability
• INF-γ secretion by ELISA
• CDR3 analysis
• TIL:Tumor co-culture
• Telomere (FlowFISH)
• Metabolic substrate analysis
• IL-2 levels by ELISA
• Immunophenotyping

Figure 9

Gen 2 Process
Gen 2.1 Process
Gen 3.1 Process
Day 0:Pre-REP
≤ 50 fragments in 1 GREX-100MCS with 1L of CM1+6,000 IU/mL IL-2
≤ 120 fragments in 3 GREX-100MCS with 1L of CM1+3,000 IU/mL IL-2
Fresh/Frozen Tumor 1-4 flask GREX-100MCS up to 60 fragments per flask 500mL of CM1+6,000 IU/mL IL-2 +OKT3 (30ng/mL) +250 E6 feeder cells
Day 11: REP-initiation
Direct to REP <200e6 TIL 1 GREX 500M in CM2+3,000 IU/mL IL-2 5E9 feeder cells 30 ng/mL OKT3 (150ul)
Direct to REP <200e6 TIL 2 one GREX 500M in CM2+3,000 IU/mL IL-2 5E9 feeder cells 30 ng/mL OKT3 (150ul)
Day 7: REP-initiation
Direct to REP – each GREX 100MCS Add 500 mL CM2+6,000 IU/mL IL-2 Feeder cells 5E8 cells/flask 30ng/mL OKT3 (30ul)
Day 16: TIL-Scale Up
Volume reduce and split up on 2 up to 5 GREX 500MCS AIMV+3,000 IU/mL of IL-2
Day 10 to 11: TIL-Scale Up
Scale up each GREX 100MCS to GREX 500MCS. Transfer all cell suspension to GREX500MCS and add 4L of CM4 + 3,000 IU/mL of IL-2
Day 22: Harvest
Harvest-LOVO automated cell washer
Day 14-16: Harvest
Harvest-LOVO automated cell washer

Figure 10

STEP	Gen 2	Gen 2.1	Gen 2.1 Optimized
Pre REP- day 0	≤ 50 fragments/ 1 G-Rex 100MCS - **11 days**	≤ 180 fragments/ 3 G-Rex, Pre-formulated CM1 warmed media 100MCS - **11 days**	***Fresh or Frozen Tumor*** Whole tumor with ≤ 30 fragments up to 60 fragments per 1 G-Rex 100MCS (up to 4 G-Rex) preformulated warmed media - **7 days. Pre REP, Feeders 2.5 E8 cells + OKT-3 (30ng/mL)**
REP Initiation	Direct to REP- Day 11- **<200 E6 TIL** 1 G-Rex 500MCS	Direct to REP- Day 11- **<200 E6 TIL** Pre-formulated CM2 warmed media in one G-Rex 500MCS	Direct to REP - Day **7-all cells TIL- same G-Rex 100MCS (100MCS up to 4 GREX), Standard media or Defined Media (Serum free). Addition Feeders 5 E8 cells +OKT-3 (30ng/mL)**
TIL propagation or Scale up	1 to 5 G-REX 500MCS **Split day 16**	2 to 5 G-REX 500MCS Pre-formulated CM4 warmed media **Split day 16**	From G-REX 100MCS transfer TIL suspension to G-REX 500MCS up to 4 GREX 500 MCS- Standard media or Defined Media (Serum Free) **Scale up on day 10 or 11**
Harvest	**Harvest day 22,** LOVO-automated cell washer	**Harvest day 22,** LOVO-automated cell washer (5 wash cycle)	**Harvest day 14 or 16** LOVO- automated cell washer (5 wash cycle)
Final formulation	Cryopreserved Product 300IU/ml IL2- CS10 in LN_2, multiple aliquots	Cryopreserved Product 300IU/ml IL2- CS10 in LN_2, multiple aliquots	Cryopreserved product 300IU/ml IL2- CS10 in LN_2, multiple aliquots
Process time	**22 days**	**22 days**	**16 days**

Figure 11

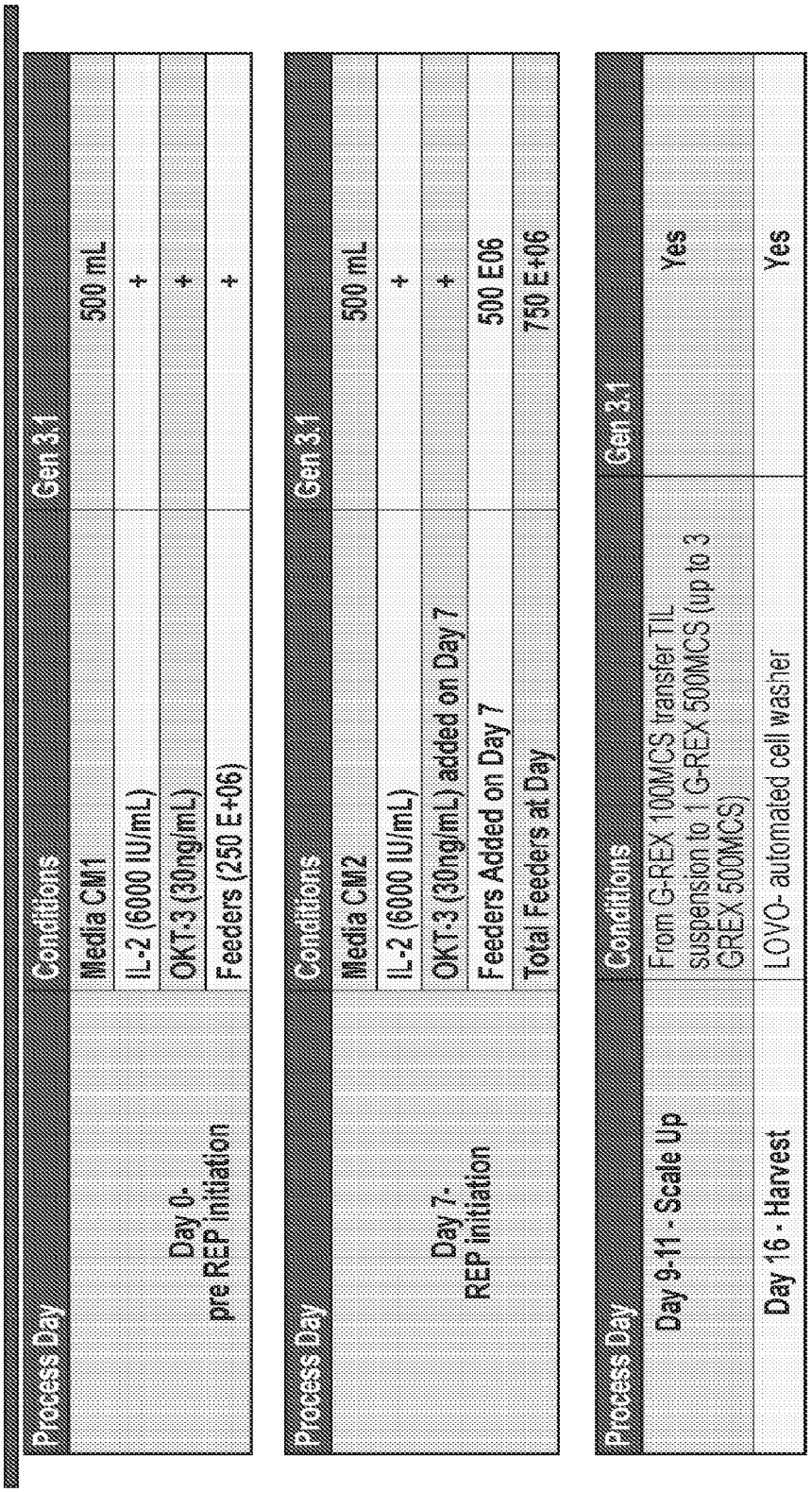
Process Day
Conditions
Gen 3.1
Day 0-
pre REP initiation
Media CM1
500 mL
IL-2 (6000 IU/mL)
+
OKT-3 (30ng/mL)
+
Feeders (250 E+06)
+
Process Day
Conditions
Gen 3.1
Day 7-
REP initiation
Media CM2
500 mL
IL-2 (6000 IU/mL)
+
OKT-3 (30ng/mL) added on Day 7
+
Feeders Added on Day 7
500 E06
Total Feeders at Day
750 E+06
Process Day
Conditions
Gen 3.1
Day 9-11 - Scale Up
From G-REX 100MCS transfer TIL suspension to 1 G-REX 500MCS (up to 3 GREX 500MCS)
Yes
Day 16 - Harvest
LOVO- automated cell washer
Yes

Figure 12

Process Comparison	Key Process Changes	Benefit
Gen 2 : Gen 2.1	• Initiate process with two flasks instead of one flask • Divide REP initiation feeder layer between 2 G-Rex500MCS Flasks • Pre-formulate media and warm prior to use	• Potential doubing of final cell count (dose) with increased TIL repertoire. • Process redundancy throughout process
Gen 2.1 : Gen 3.1	• Fresh or Frozen tumor • 14-16 day process (from 22 day) • Reduce total feeder layer on process • Feeder layer and OKT3 present at Day 0 • REP initiated with fragments • 100MCS scales to 500MCS • Scales to multiple pre-REP flasks • Standard Media and Defined Media (Serum Free)	• Increased potency • Improved phenotype • Decreased process time • Reduced reagent testing • Decreased process variability • Defined reagents • Increased repertoire • Reduce impurities (feeder) • Comparable or Higher Dose

Figure 13

Process Comparison	Key Process Changes	Desired Improvement	Criteria for Success	Outcome
Gen 2 : Gen 3.0	• 14-16 days • Initiate REP with fragments up to 4 flask. • 100MCS scales to 500MCS	• Increased potency • Improved phenotype • Decreased process time	• Increase potency as measured by INF-g ✓ • Comparable phenotype ✓ • Comparable Dose ✓ • Comparable purity (feeder cell) ✓ • Maintain clonal diversity ✓	• Potency increased over Gen2 • Improved expression of CD28 on CD8 cells • Maximum capacity of flask reached by day 16 on Gen 3.1 • Reduced feeder cell usage • Increased diversity

Figure 14

Process	Gen 2	Gen 3
L4054	Standard Media	Standard Media
L4055	Standard Media	Standard Media
M1085T	Standard Media	Standard Media

Standard Media:
Pre REP: CM1
REP initiation : CM2
Split or Scale up : CM4

Process	Gen 3	Gen 3.1 control	Gen 3.1
L4063	Standard Media	Standard Media	Standard Media
L4064	Defined Media	Defined Media	Defined Media

Defined Media:
CTS Optimizer (Serum Free Media) in each day of the process

Figure 15

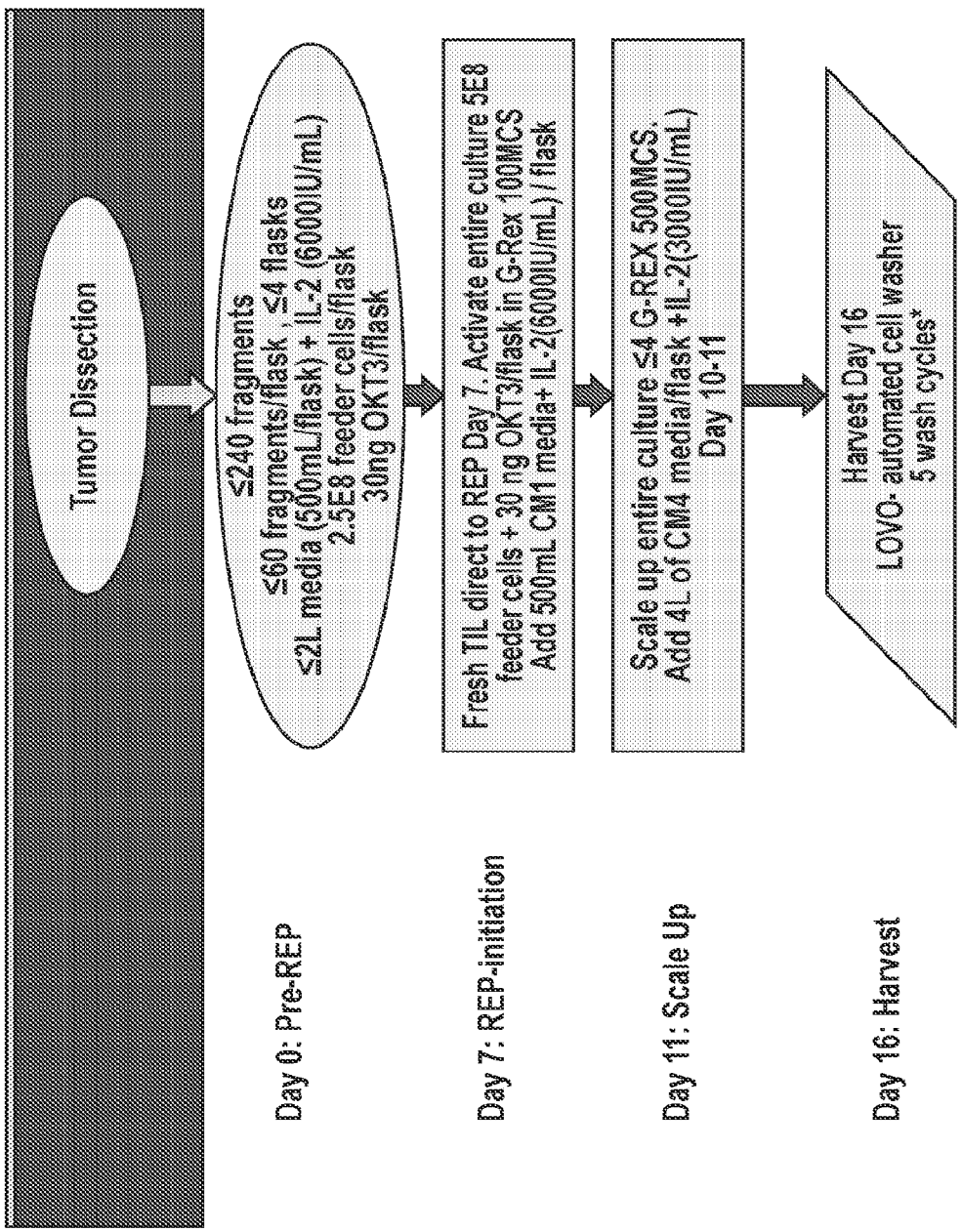
Tumor Dissection
≤240 fragments
≤60 fragments/flask , ≤4 flasks
≤2L media (500mL/flask) + IL-2 (6000IU/mL)
2.5E8 feeder cells/flask
30ng OKT3/flask
Fresh TIL direct to REP Day 7, Activate entire culture 5E8
feeder cells + 30 ng OKT3/flask in G-Rex 100MCS
Add 500mL CM1 media+ IL-2(6000IU/mL) / flask
Scale up entire culture ≤4 G-REX 500MCS.
Add 4L of CM4 media/flask +IL-2(3000IU/mL)
Day 10-11
Harvest Day 16
LOVO- automated cell washer
5 wash cycles*
Day 0: Pre-REP
Day 7: REP-initiation
Day 11: Scale Up
Day 16: Harvest

Figure 16

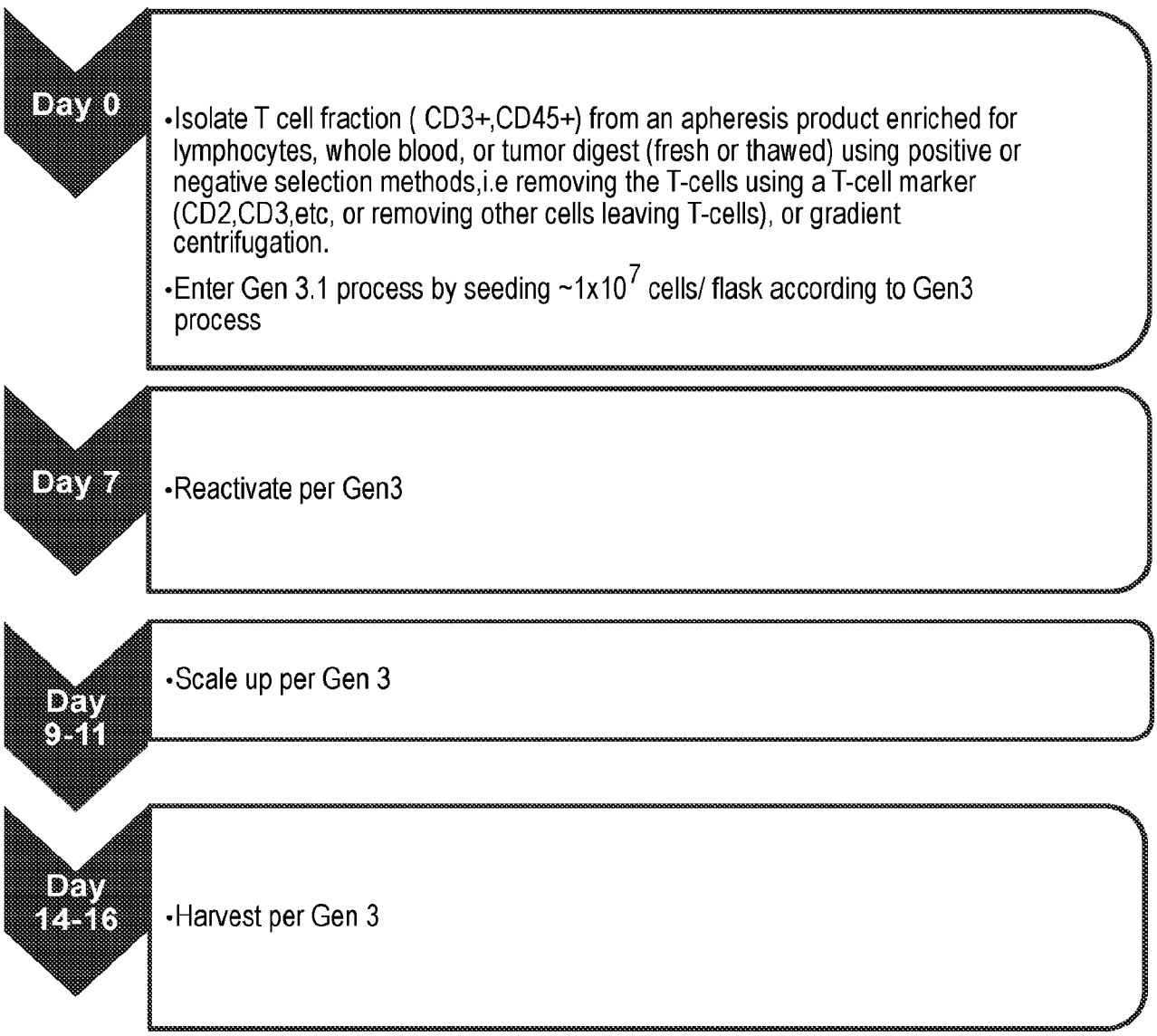
Day 0
•Isolate T cell fraction (CD3+,CD45+) from an apheresis product enriched for lymphocytes, whole blood, or tumor digest (fresh or thawed) using positive or negative selection methods,i.e removing the T-cells using a T-cell marker (CD2,CD3,etc, or removing other cells leaving T-cells), or gradient centrifugation.
•Enter Gen 3.1 process by seeding ~1x10^7 cells/ flask according to Gen3 process
Day 7
•Reactivate per Gen3
Day 9-11
•Scale up per Gen 3
Day 14-16
•Harvest per Gen 3

Figure 17

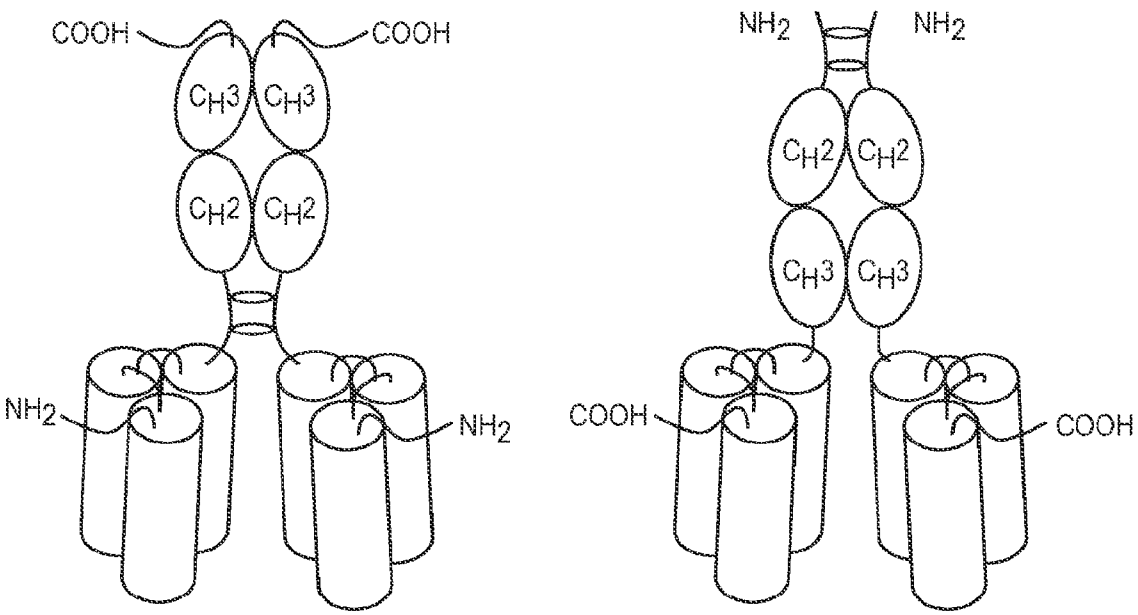
COOH
COOH
C_H3
C_H3
C_H2
C_H2
NH_2
NH_2
Structure I-A
NH_2
NH_2
C_H2
C_H2
C_H3
C_H3
COOH
COOH
Structure I-B

Figure 18

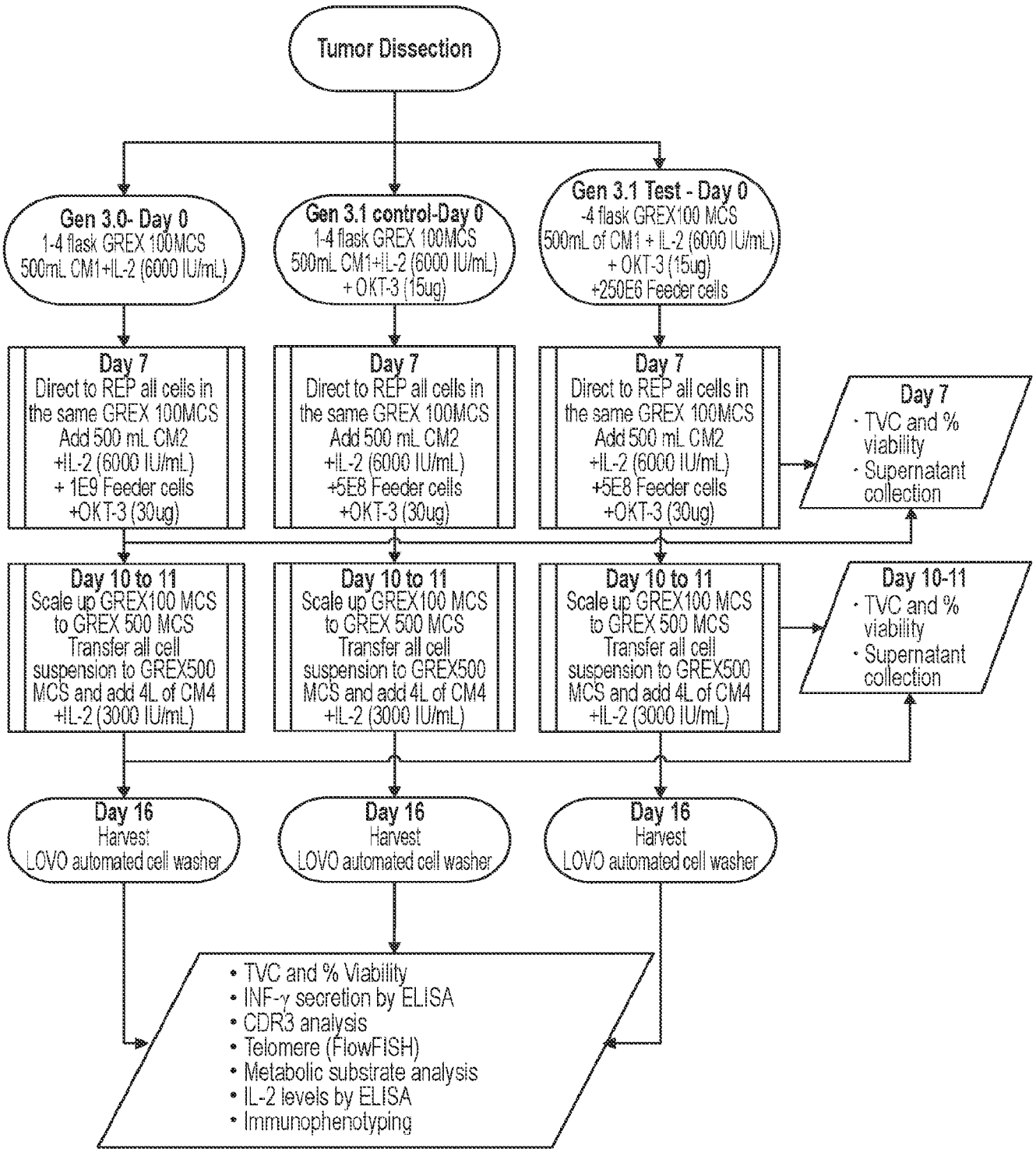
Tumor Dissection
Gen 3.0- Day 0
1-4 flask GREX 100MCS
500mL CM1+IL-2 (6000 IU/mL)
Gen 3.1 control-Day 0
1-4 flask GREX 100MCS
500mL CM1+IL-2 (6000 IU/mL)
+ OKT-3 (15ug)
Gen 3.1 Test - Day 0
-4 flask GREX100 MCS
500mL of CM1 + IL-2 (6000 IU/mL)
+ OKT-3 (15ug)
+250E6 Feeder cells
Day 7
Direct to REP all cells in the same GREX 100MCS
Add 500 mL CM2
+IL-2 (6000 IU/mL)
+ 1E9 Feeder cells
+OKT-3 (30ug)
Day 7
Direct to REP all cells in the same GREX 100MCS
Add 500 mL CM2
+IL-2 (6000 IU/mL)
+5E8 Feeder cells
+OKT-3 (30ug)
Day 7
Direct to REP all cells in the same GREX 100MCS
Add 500 mL CM2
+IL-2 (6000 IU/mL)
+5E8 Feeder cells
+OKT-3 (30ug)
Day 7
· TVC and % viability
· Supernatant collection
Day 10 to 11
Scale up GREX100 MCS to GREX 500 MCS
Transfer all cell suspension to GREX500 MCS and add 4L of CM4
+IL-2 (3000 IU/mL)
Day 10 to 11
Scale up GREX100 MCS to GREX 500 MCS
Transfer all cell suspension to GREX500 MCS and add 4L of CM4
+IL-2 (3000 IU/mL)
Day 10 to 11
Scale up GREX100 MCS to GREX 500 MCS
Transfer all cell suspension to GREX500 MCS and add 4L of CM4
+IL-2 (3000 IU/mL)
Day 10-11
· TVC and % viability
· Supernatant collection
Day 16
Harvest
LOVO automated cell washer
Day 16
Harvest
LOVO automated cell washer
Day 16
Harvest
LOVO automated cell washer
• TVC and % Viability
• INF-γ secretion by ELISA
• CDR3 analysis
• Telomere (FlowFISH)
• Metabolic substrate analysis
• IL-2 levels by ELISA
• Immunophenotyping

Figure 19

Process Day	Conditions	Gen 3.1 Test
Day 0- pre REP initiation	Media CM1	500 mL
	IL-2 (6000 IU/mL)	+
	OKT-3 (15ug)	+
	Feeders (250 E+06)	+

Process Day	Conditions	Gen 3.1 Test
Day 7- REP initiation	Media CM2	500 mL
	IL-2 (6000 IU/mL)	+
	OKT-3 (30ug) added on Day 7	+
	Feeders Added on Day 7	500 E06
	Total Feeders at Day	750 E+06

Process Day	Conditions	Gen 3.1 Test
Day 9-11 - Scale Up	From G-REX 100MCS transfer TIL suspension to 1 G-REX 500MCS (up to 3 GREX 500MCS)	Yes
Day 16 - Harvest	LOVO- automated cell washer	Yes

Figure 20

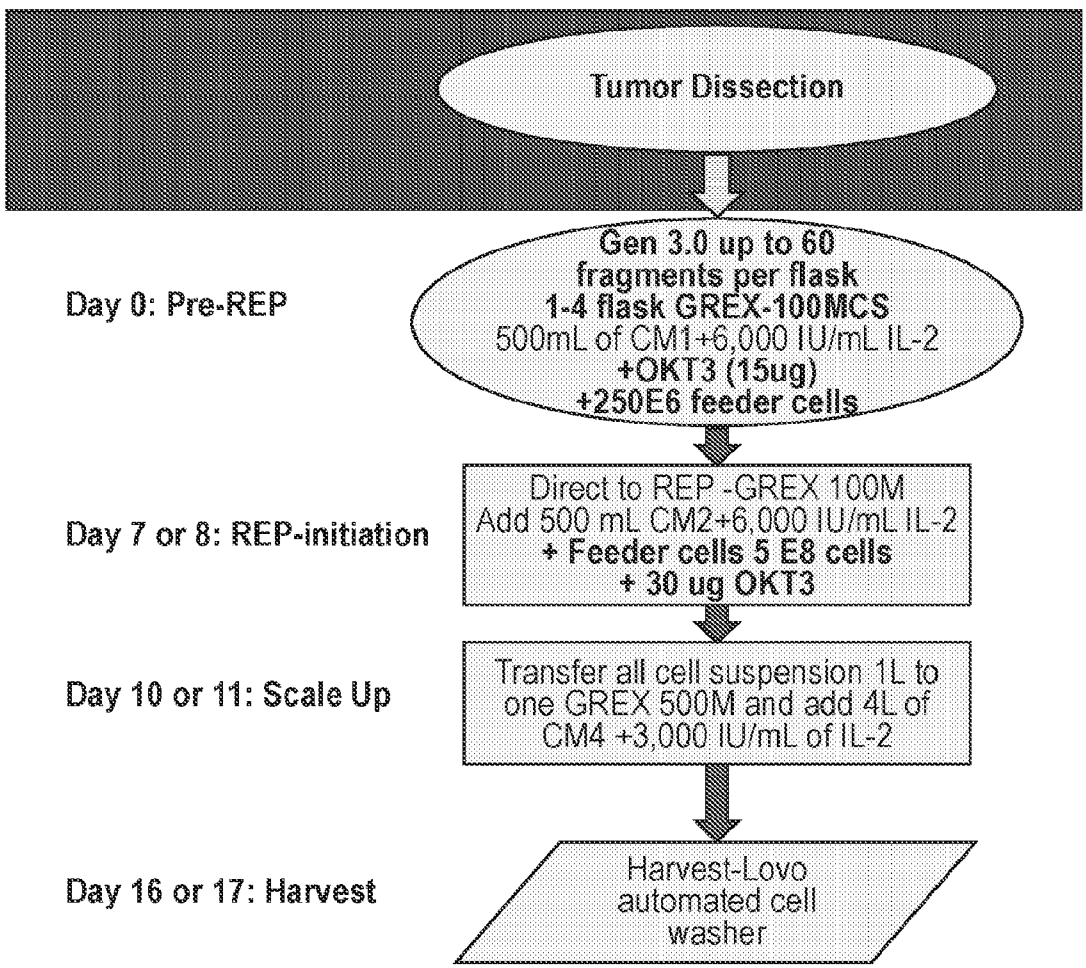
Tumor Dissection
Day 0: Pre-REP
Gen 3.0 up to 60 fragments per flask
1-4 flask GREX-100MCS
500mL of CM1+6,000 IU/mL IL-2
+OKT3 (15ug)
+250E6 feeder cells
Day 7 or 8: REP-initiation
Direct to REP -GREX 100M
Add 500 mL CM2+6,000 IU/mL IL-2
+ Feeder cells 5 E8 cells
+ 30 ug OKT3
Day 10 or 11: Scale Up
Transfer all cell suspension 1L to one GREX 500M and add 4L of CM4 +3,000 IU/mL of IL-2
Day 16 or 17: Harvest
Harvest-Lovo automated cell washer

Figure 21

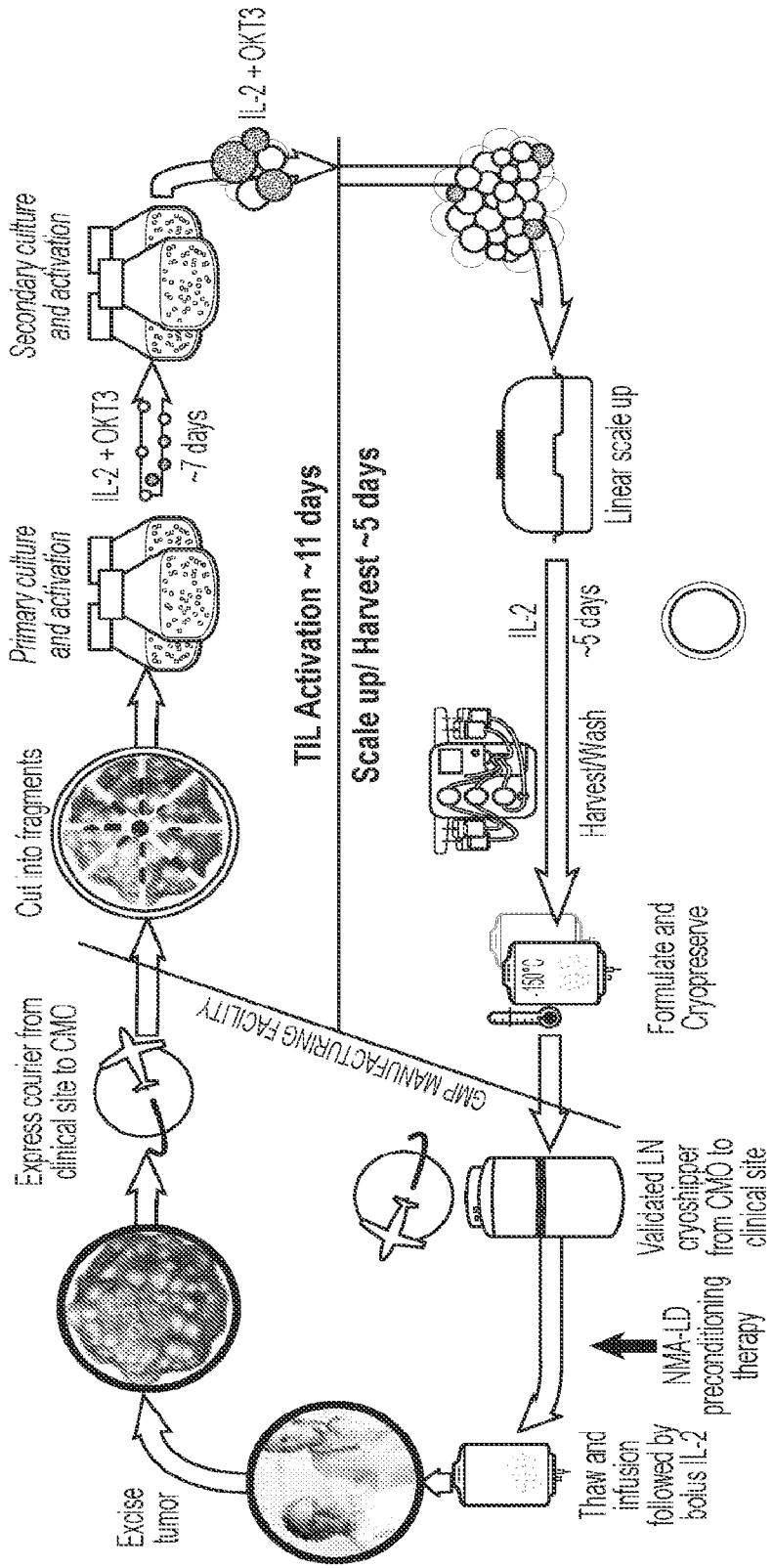
Excise tumor
Express courier from clinical site to CMO
Cut into fragments
Primary culture and activation
IL-2 + OKT3
~7 days
Secondary culture and activation
IL-2 + OKT3
TIL Activation ~11 days
Scale up/ Harvest ~5 days
GMP MANUFACTURING FACILITY
Linear scale up
IL-2
~5 days
Harvest/Wash
Formulate and Cryopreserve
Validated LN cryoshipper from CMO to clinical site
NMA-LD preconditioning therapy
Thaw and infusion followed by bolus IL-2

Figure 22

	Gen 2	Gen 3
Total culture time	22 days	16-17 days (or 16 days)
Pre-REP		
Fragments/flask	≤ 60 fragments in one flask	≤ 60 fragments in up to 4 flasks
Media volume	1L – single addition	1L – 2 x 500 mL additions
Target pre-REP cell numbers	<200 x 10^6 TIL	All cells carried through continuous process
Screening	No screen	No screen
Selection of flasks	No selection	Bact-T sterility, visual inspection for contaminants
REP/Scale up		
Feeders		Reduced by ≥ 40%
Media	Contains HSAB	Defined medium
Scale up	Pooled culture; volume reduce to 500 mL on Day 5 split up to 5 flasks (2500 cm^2)	Flasks scaled linearly and treated as subcomponents
OKT3	150 μg	≤ 180 μg
IL-2	High dose	High dose
Number of flasks	1-5	1-4
Final steps		
Harvest/volume reduction	Closed 10:1	Closed 10:1
Concentrate/wash	LOVO 100:1	LOVO 1000:1
Formulation conditions	1:1 CS10 (5% DMSO)	1:1 CS10 (5% DMSO)
Shipment conditions	Vapor phase liquid nitrogen	Vapor phase liquid nitrogen
Infusion	Thawed IV gravity	Thawed IV gravity

Figure 23

LD on:
Day 10-11 Viability
Day 10-11 Phenotype
Day 10-11 myco
Day 10-11 IFNg*
Release for infusion on:
Gram Stain
Day 10-11 Sterility
Day 16-17 Mycoplasma
Day 16-17 Endotoxin
Day 16-17 Phenotyping
Day 16-17 TVC count
Day 16-17 Viability
Final Release (CoA)
Day 16-17 Frozen/Thawed IFNg (ELISA)
Day 16-17 Endotoxin
Day 16-17 Phenotyping
Day 16-17 TVC count
Day 16-17 Viability
Day 16-17 Sterility
* Restimulation for ELISA on Day 10-11
will be from a fresh / frozen in-process sample reportable
On Day 14
Lymphodepletion
IFN-g results
Final Sterility results
Surgery
Ship
Infuse
0
7
11
17
21
24
Culture Initiation
In-Process Testing (Visual inspection, Sterility, Myco, Phenotype, IFNg)
Harvest (Sterility, TVC, Viability, Mycoplasma, Phenotype, Gram Stain, Endotoxin, IFNg)
TIL Activation
Scale Up

Figure 24

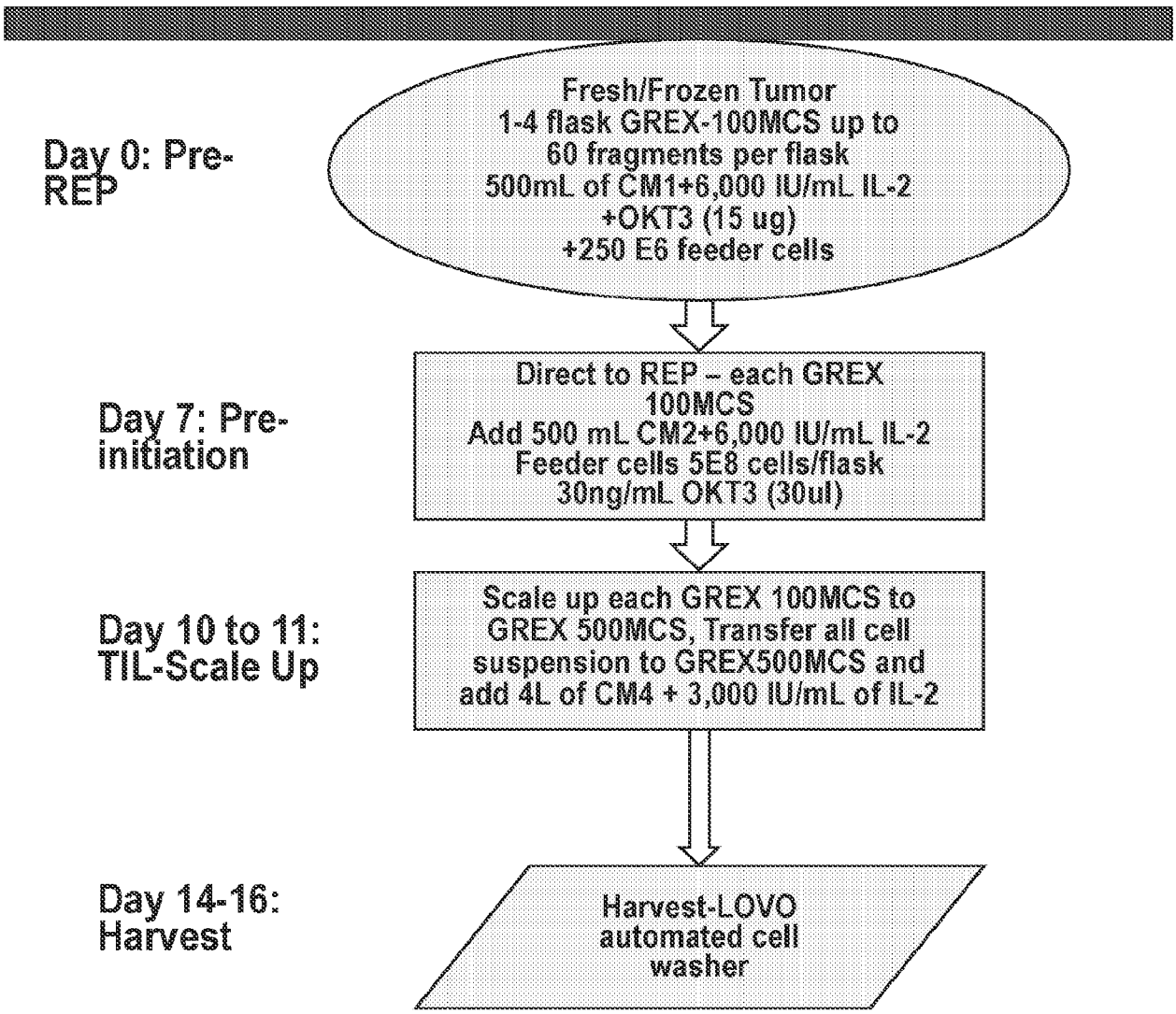
Day 0: Pre-REP
Fresh/Frozen Tumor
1-4 flask GREX-100MCS up to
60 fragments per flask
500mL of CM1+6,000 IU/mL IL-2
+OKT3 (15 ug)
+250 E6 feeder cells
Day 7: Pre-initiation
Direct to REP – each GREX 100MCS
Add 500 mL CM2+6,000 IU/mL IL-2
Feeder cells 5E8 cells/flask
30ng/mL OKT3 (30ul)
Day 10 to 11: TIL-Scale Up
Scale up each GREX 100MCS to GREX 500MCS, Transfer all cell suspension to GREX500MCS and add 4L of CM4 + 3,000 IU/mL of IL-2
Day 14-16: Harvest
Harvest-LOVO automated cell washer

Figure 25

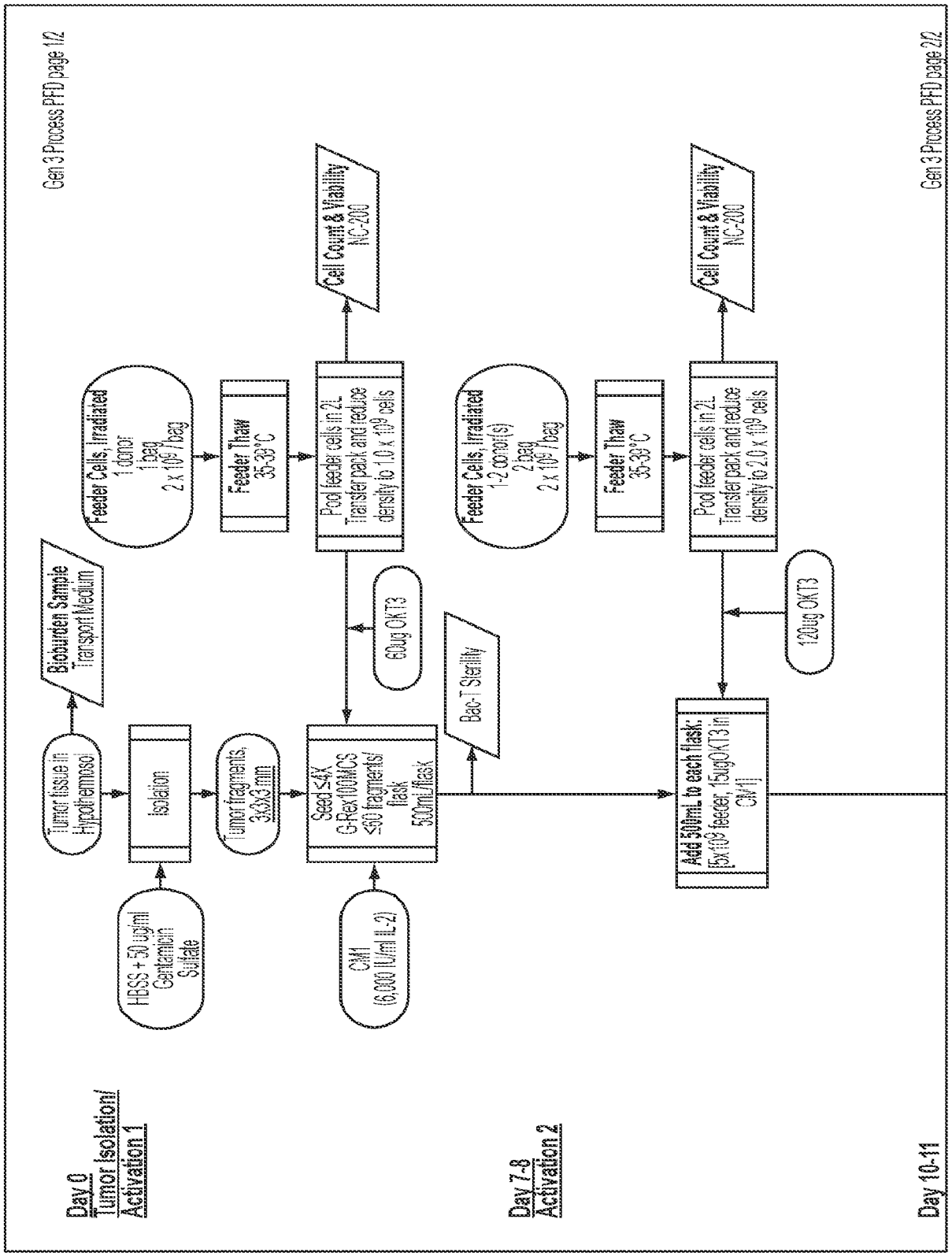
Gen 3 Process PFD page 1/2
Day 0
Tumor Isolation/
Activation 1
Tumor tissue in Hypothermosol
Bioburden Sample
Transport Medium
Isolation
HBSS + 50 ug/ml Gentamicin Sulfate
Tumor fragments, 3x3x3 mm
Seed ≤4X
G-Rex100MCS
≤50 fragments/
flask
500ml/flask
CM1
(6,000 IU/ml IL-2)
60ug OKT3
Bac-T Sterility
Feeder Cells, Irradiated
1 donor
1 bag
2 x 10^9 /bag
Feeder Thaw
35-39°C
Pool feeder cells in 2L
Transfer pack and reduce
density to 1.0 x 10^9 cells
Cell Count & Viability
NC-200
Day 7-8
Activation 2
Feeder Cells, Irradiated
1-2 donor(s)
2 bag
2 x 10^9 /bag
Feeder Thaw
35-39°C
Pool feeder cells in 2L
Transfer pack and reduce
density to 2.0 x 10^9 cells
Cell Count & Viability
NC-200
120ug OKT3
Add 500mL to each flask:
[5x10^9 feeder, 15ug OKT3 in
CM1]
Day 10-11
Gen 3 Process PFD page 2/2

Figure 26A

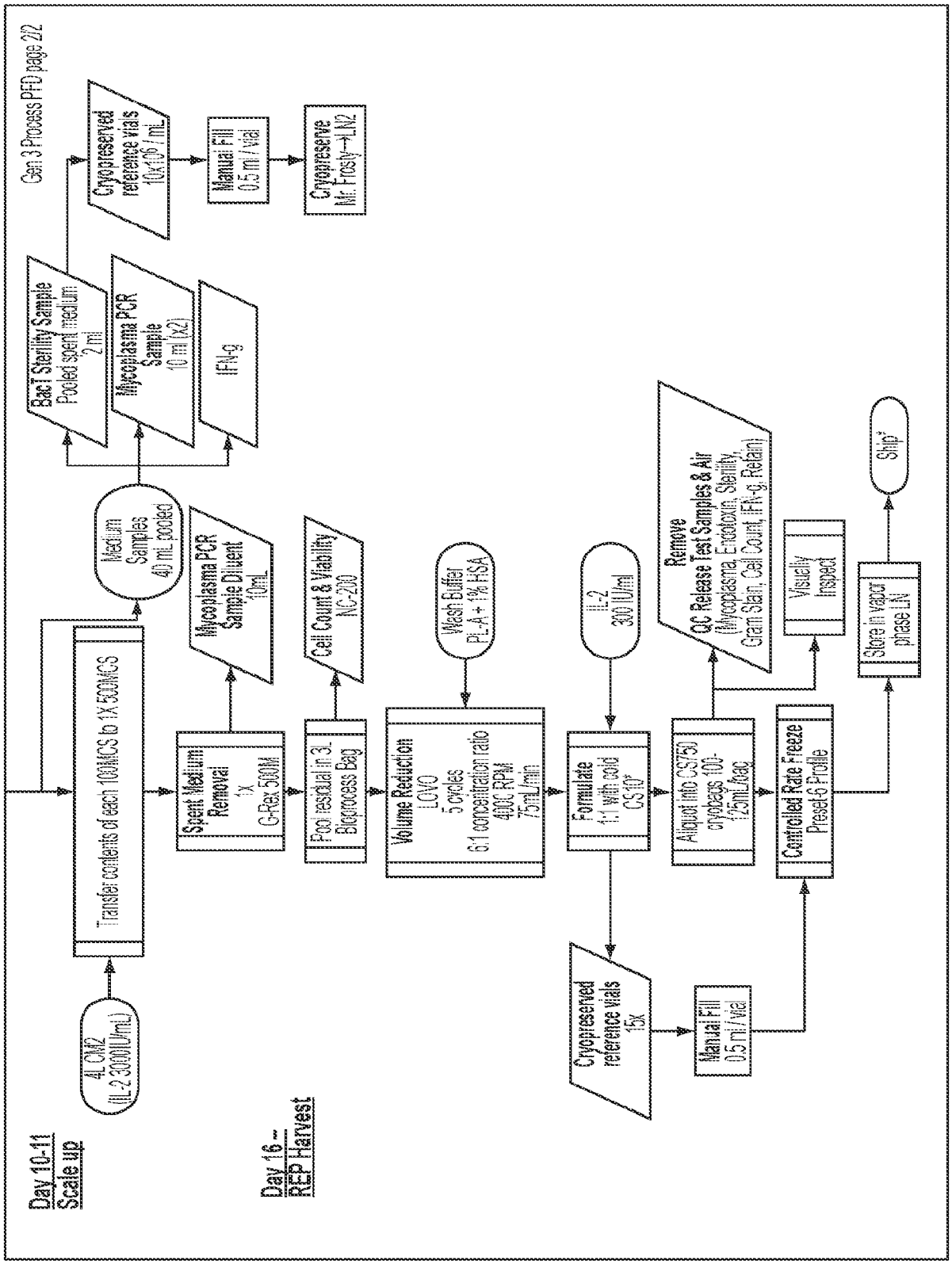
Gen 3 Process PFD page 2/2
Day 10-11
Scale up
4L CM2
(IL-2 3000IU/mL)
Transfer contents of each 100MCS to 1X 500MCS
Day 16 –
REP Harvest
Spent Medium Removal
1X
G-Rex 500M
Pool residual in 3L
Bioprocess Bag
Volume Reduction
LOVO
5 cycles
6:1 concentration ratio
4000 RPM
75mL/min
Formulate
1:1 with cold
CS10*
Aliquot into CS750
cryobags 100-
125mL/bag
Controlled Rate Freeze
Preset-6 Profile
Store in vapor
phase LN
Ship*
Medium
Samples
40 mL pooled
Mycoplasma PCR
Sample Diluent
10mL
Cell Count & Viability
NC-200
Wash Buffer
PL-A + 1% HSA
IL-2
300 IU/ml
Remove
QC Release Test Samples & Air
(Mycoplasma, Endotoxin, Sterility,
Gram Stain, Cell Count, IFN-g, Retain)
Visually
Inspect
Bact Sterility Sample
Pooled spent medium
2 ml
Mycoplasma PCR
Sample
10 ml (x2)
IFN-g
Cryopreserved
reference vials
10x10^6 / mL
Manual Fill
0.5 ml / vial
Cryopreserve
Mr. Frosty→LN2
Cryopreserved
reference vials
15x
Manual Fill
0.5 ml / vial

Figure 26B

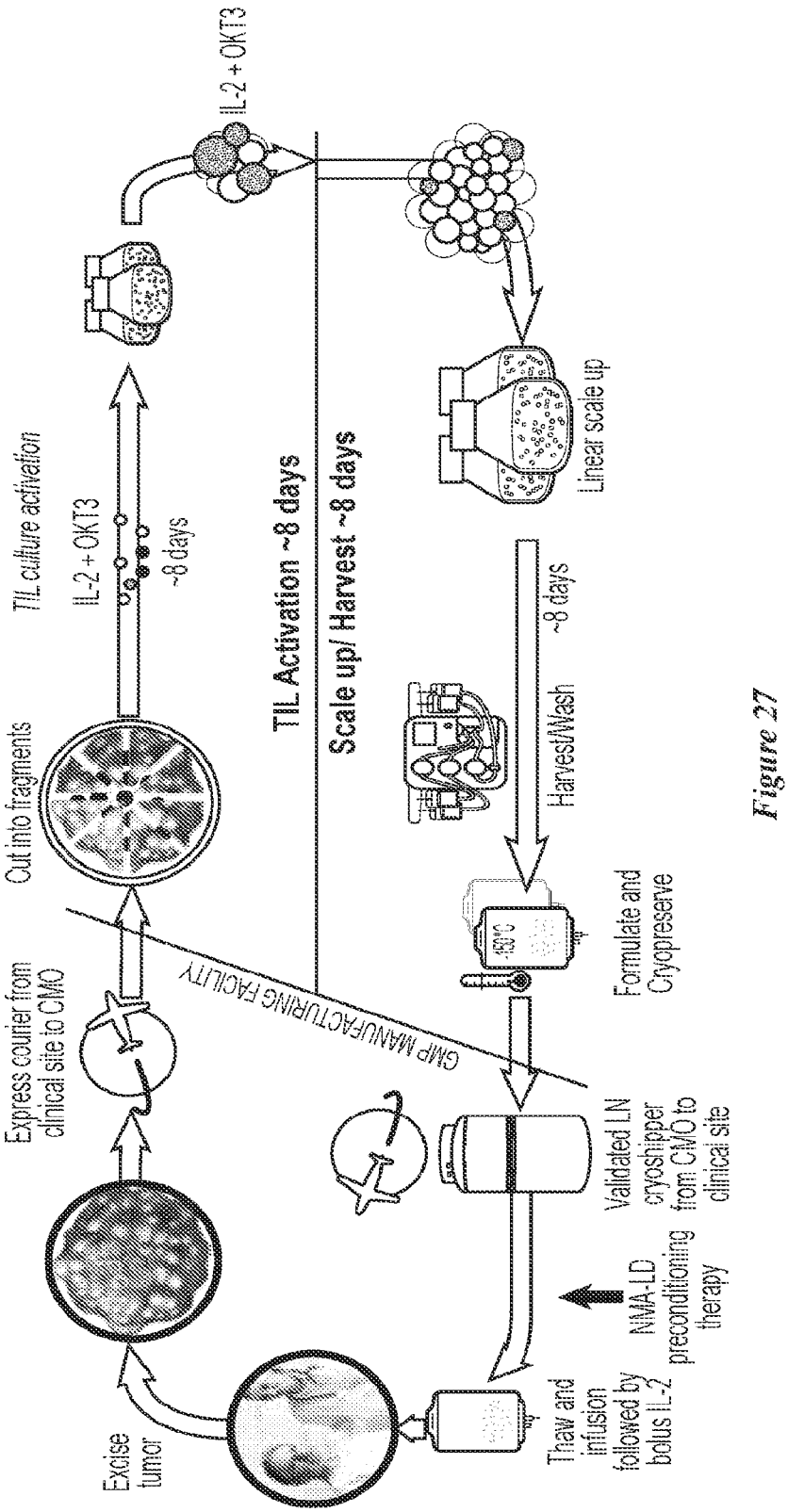
Excise tumor
Thaw and infusion followed by bolus IL-2
NMA-LD preconditioning therapy
Validated LN cryoshipper from CMO to clinical site
Express courier from clinical site to CMO
GMP MANUFACTURING FACILITY
Out into fragments
TIL culture activation
IL-2 + OKT3
~8 days
TIL Activation ~8 days
Scale up/ Harvest ~8 days
IL-2 + OKT3
Linear scale up
~8 days
Harvest/Wash
Formulate and Cryopreserve

Figure 27

STEP	Gen 2	Gen 2.1	Gen 3.1a
Pre REP- day 0	≤ 50 fragments/ 1 G-Rex 100MCS - **11 days**	≤ 180 fragments/ 3 G-Rex, Pre-formulated CM1 warmed media 100MCS - **11 days**	***Fresh or Frozen Tumor*** Whole tumor with ≤ 30 fragments up to 60 fragments per 1 G-Rex 100MCS (up to 4 G-Rex) preformulated warmed media - **7 days. Pre REP, Feeders 250e^6 cells + OKT-3 (15ug)**
REP Initiation	Direct to REP- Day 11- **<200e^6 TIL** 1 G-Rex 500MCS	Direct to REP- Day 11- **<200e^6 TIL** Pre-formulated CM2 warmed media in one G-Rex 500MCS	Direct to REP - Day **7-all cells TIL- same G-Rex 100MCS (100MCS up to 4 GREX), Standard media or Defined Media (Serum free). Addition Feeders 500e^6 cells +OKT-3 (30ug)**
TIL propagation or Scale up	1 to 5 G-REX 500MCS **Split day 16**	2 to 5 G-REX 500MCS Pre-formulated CM4 warmed media **Split day 16**	From G-REX 100MCS transfer TIL suspension to G-REX 500MCS up to 4 GREX 500 MCS- Standard media or Defined Media (Serum Free) **Scale up on day 10 or 11**
Harvest	**Harvest day 22,** LOVO-automated cell washer	**Harvest day 22,** LOVO-automated cell washer (5 wash cycle)	**Harvest day 14 or 16** LOVO- automated cell washer (5 wash cycle)
Final formulation	Cryopreserved Product 300IU/ml IL2- CS10 in LN_2, multiple aliquots	Cryopreserved Product 300IU/ml IL2- CS10 in LN_2, multiple aliquots	Cryopreserved product 300IU/ml IL2- CS10 in LN_2, multiple aliquots
Process time	**22 days**	**22 days**	**16 days**

Figure 28

Process Comparison	Process Changes	Differences
Gen 2 : Gen 2.1	• Initiate process with two flasks instead of one flask • Divide REP initiation feeder layer between 2 G-Rex500MCS Flasks • Pre-formulate media and warm prior to use	• Potential doubing of final cell count (dose with increased TIL repertoire. • Process redundancy throughout process
Gen 2.1 : Gen 3.1	• Fresh or Frozen tumor • 14-16 day process (from 22 day) • Reduce total feeder layer on process • Feeder layer and OKT3 present at Day 0 • REP initiated with fragments • 100MCS scales to 500MCS • Scales to multiple pre-REP flasks • Standard Media and Defined Media (Serum Free)	• Increased potency • Improved phenotype • Decreased process time • Reduced reagent testing • Decreased process variability • Defined reagents • Increased repertoire • Reduce impurities (feeder) • Comparable or Higher Dose

Figure 29

Process Day	Conditions	Gen 3.0	Gen 3.1 control	Gen 3.1 Test
Day 0 : Tumor Fragment Isolation and Activation	Media (*)	500 mL	500 mL	500 mL
	IL-2	6000 IU/mL	6000 IU/mL	6000 IU/mL
	OKT-3	-	15 ug	15 ug
	Feeders	-	-	2.5E+06
Process Day	Conditions	Gen 3.0	Gen 3.1 control	Gen 3.1 Test
Day 7 - 8 : TIL Culture Reactivation	Media (*)	500 mL	500 mL	500 mL
	IL-2	6000 IU/mL	6000 IU/mL	6000 IU/mL
	OKT-3	30 ug	30 ug	30 ug
	Feeders	1 E+09	500 E+06	500E+06
	Total Feeders added through Day 7	1 E+09	500 E+06	750E+06
Process Day	Conditions	Gen 3.0	Gen 3.1 control	Gen 3.1 Test
Day 10-11 : Culture Scale Up	From GREX 100 transfer whole TIL suspension to 1 GREX 500 containing 4L media with IL-2 (3000 IU/mL)			
Process Day	Conditions	Gen 3.0	Gen 3.1 control	Gen 3.1 Test
Day 16-17: Harvest/Wash/Formulate	LOVO automated cell washer and cryopreservation with CS10.			

(*) Media can be standard media or CTS serum free media.

Figure 30

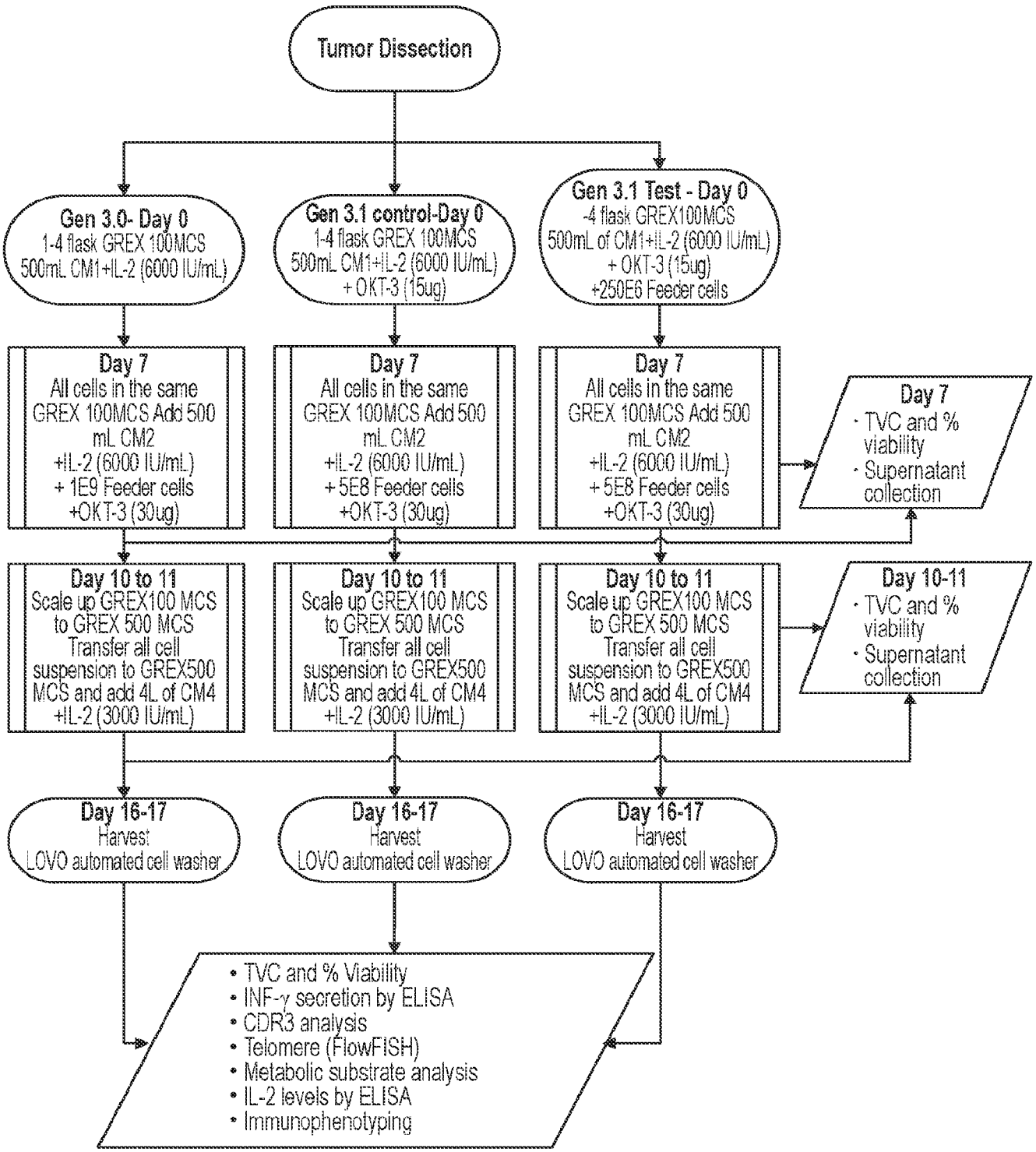
Tumor Dissection
Gen 3.0- Day 0
1-4 flask GREX 100MCS
500mL CM1+IL-2 (6000 IU/mL)
Gen 3.1 control-Day 0
1-4 flask GREX 100MCS
500mL CM1+IL-2 (6000 IU/mL)
+ OKT-3 (15ug)
Gen 3.1 Test - Day 0
-4 flask GREX100MCS
500mL of CM1+IL-2 (6000 IU/mL)
+ OKT-3 (15ug)
+250E6 Feeder cells
Day 7
All cells in the same GREX 100MCS Add 500 mL CM2
+IL-2 (6000 IU/mL)
+ 1E9 Feeder cells
+OKT-3 (30ug)
Day 7
All cells in the same GREX 100MCS Add 500 mL CM2
+IL-2 (6000 IU/mL)
+ 5E8 Feeder cells
+OKT-3 (30ug)
Day 7
All cells in the same GREX 100MCS Add 500 mL CM2
+IL-2 (6000 IU/mL)
+ 5E8 Feeder cells
+OKT-3 (30ug)
Day 7
· TVC and % viability
· Supernatant collection
Day 10 to 11
Scale up GREX100 MCS to GREX 500 MCS
Transfer all cell suspension to GREX500 MCS and add 4L of CM4
+IL-2 (3000 IU/mL)
Day 10 to 11
Scale up GREX100 MCS to GREX 500 MCS
Transfer all cell suspension to GREX500 MCS and add 4L of CM4
+IL-2 (3000 IU/mL)
Day 10 to 11
Scale up GREX100 MCS to GREX 500 MCS
Transfer all cell suspension to GREX500 MCS and add 4L of CM4
+IL-2 (3000 IU/mL)
Day 10-11
· TVC and % viability
· Supernatant collection
Day 16-17
Harvest
LOVO automated cell washer
Day 16-17
Harvest
LOVO automated cell washer
Day 16-17
Harvest
LOVO automated cell washer
• TVC and % Viability
• INF-γ secretion by ELISA
• CDR3 analysis
• Telomere (FlowFISH)
• Metabolic substrate analysis
• IL-2 levels by ELISA
• Immunophenotyping

Figure 31

Step	Process Gen 3-Optimized
Day 0 Tumor isolation and Activation	≤240 fragments ≤60 fragments/flask ≤4 flasks ≤2L media (500mL/flask) IL-2 (6000IU/mL) $2.5x10^8$ feeder cells/flask 15ug OKT3/flask
Day 7 - 8 Reactivation	Fresh TIL direct to REP Activate entire culture $5x10^8$ feeder cells 30 ug OKT3/flask G-Rex 100MCS Add 500mL media+ IL-2(6000IU/mL)
Day 10 - 11 Scale up or TIL Sub-culture	≤4 G-REX 500MCS Scale up entire culture transferring 1L from GREX 100MCS into GREX 500MCS and add 4L of media +IL-2 (3000 IU/mL) /flask
Day 16 - 17 Harvest	Harvest LOVO- automated cell washer Cryopreservation on Plasmalyte 1% HSA: CS10

Figure 32

Test	Acceptance Criteria	Gen 3.1 Test vs Gen 3.0 Process
Cell Count (TVC)	Gen 3.1 > 30% to Process Gen 3.0	Met
% Viability	≥70% Viability	Met
Immunophenotyping (%CD3+/ %CD45+)	≤5% difference between Gen 3.1 and Gen 3.0 process	Met
IFNγ secretion	Gen 3.1 ≥ to Process Gen 3.0	Met

Figure 33

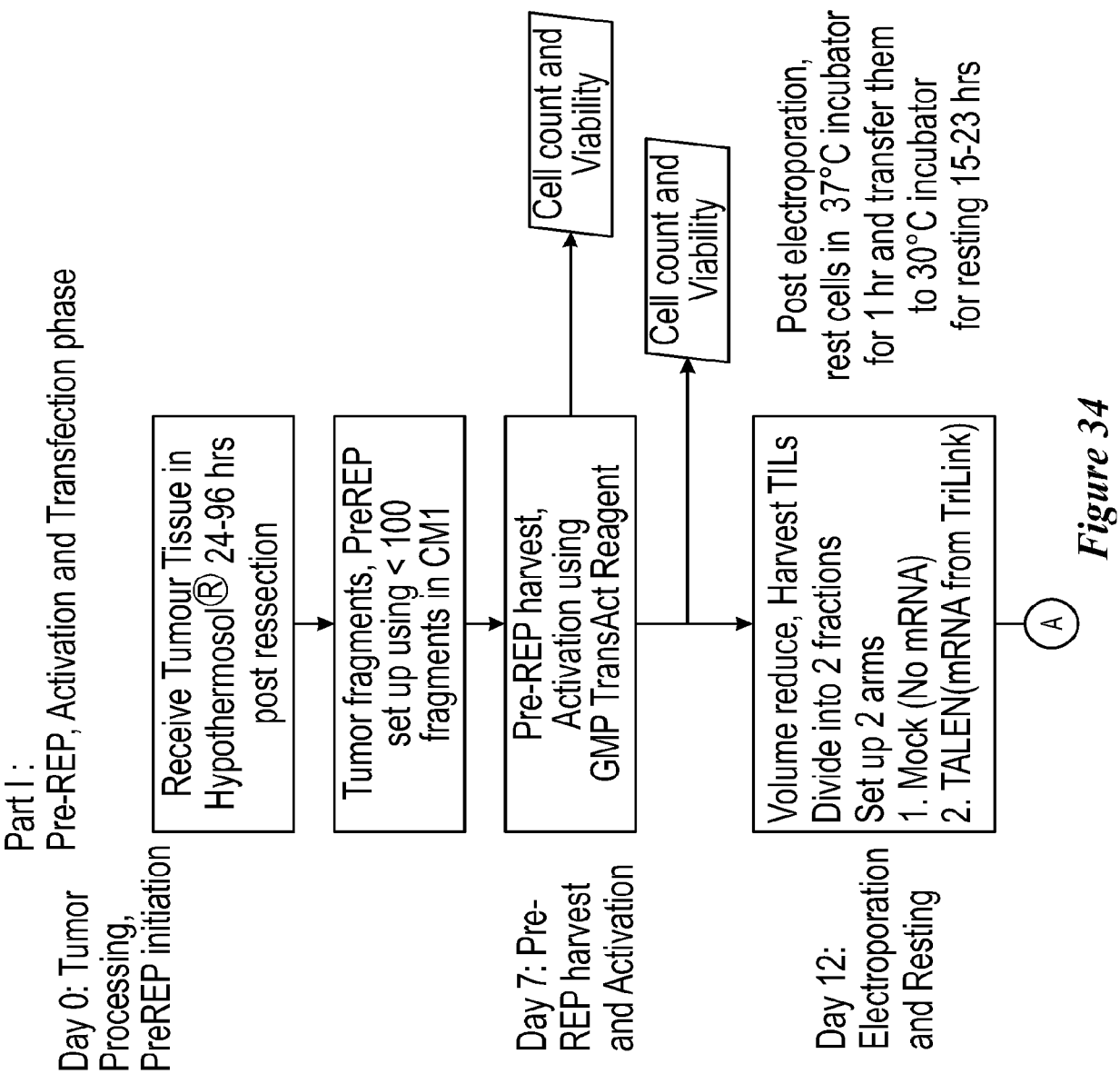
Part I :
Pre-REP, Activation and Transfection phase
Day 0: Tumor Processing, PreREP initiation
Receive Tumour Tissue in Hypothermosol® 24-96 hrs post ressection
Tumor fragments, PreREP set up using < 100 fragments in CM1
Day 7: Pre-REP harvest and Activation
Pre-REP harvest, Activation using GMP TransAct Reagent
Cell count and Viability
Cell count and Viability
Day 12: Electroporation and Resting
Volume reduce, Harvest TILs
Divide into 2 fractions
Set up 2 arms
1. Mock (No mRNA)
2. TALEN(mRNA from TriLink)
A
Post electroporation, rest cells in 37°C incubator for 1 hr and transfer them to 30°C incubator for resting 15-23 hrs

Figure 34

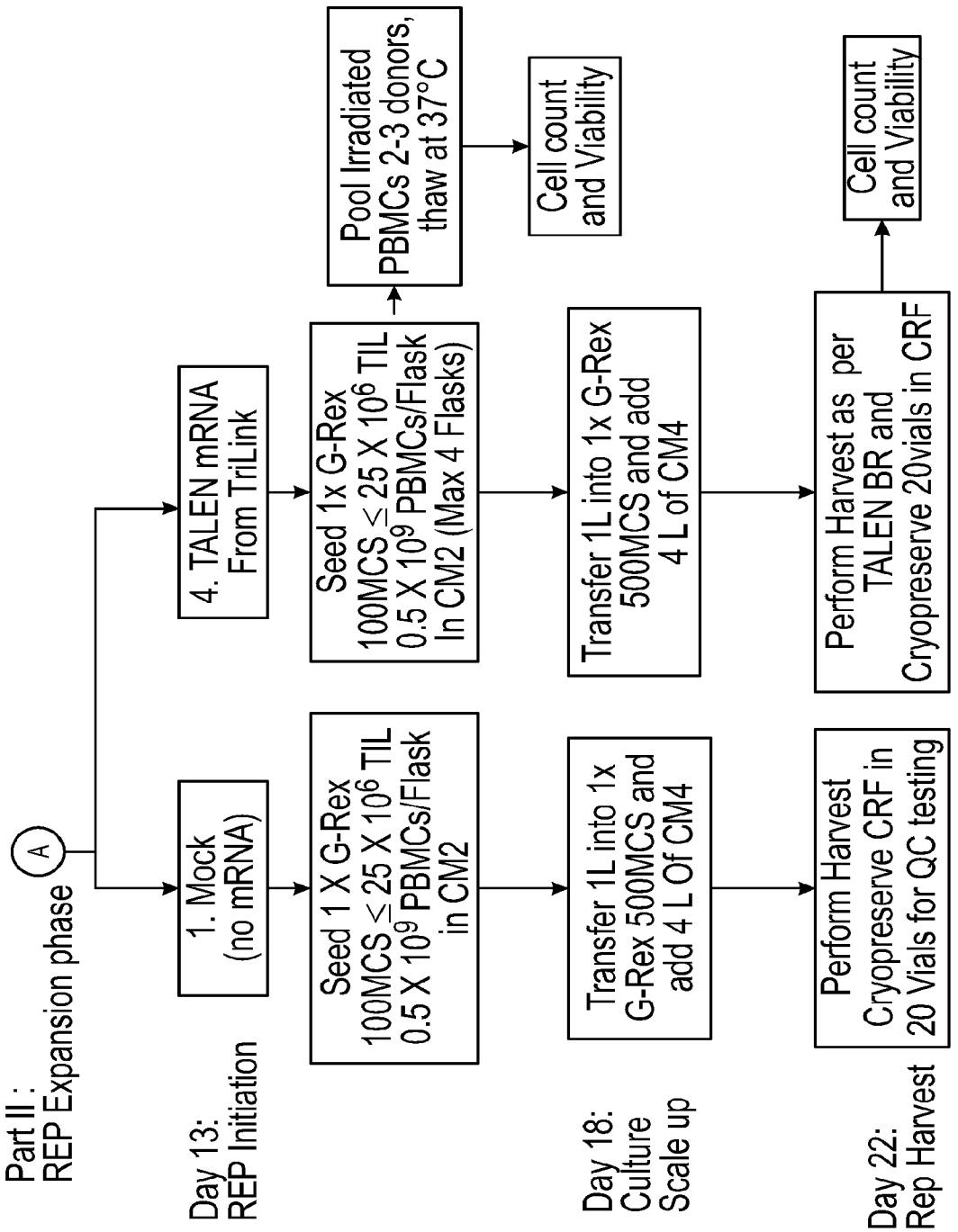

Figure 34
(Continued)

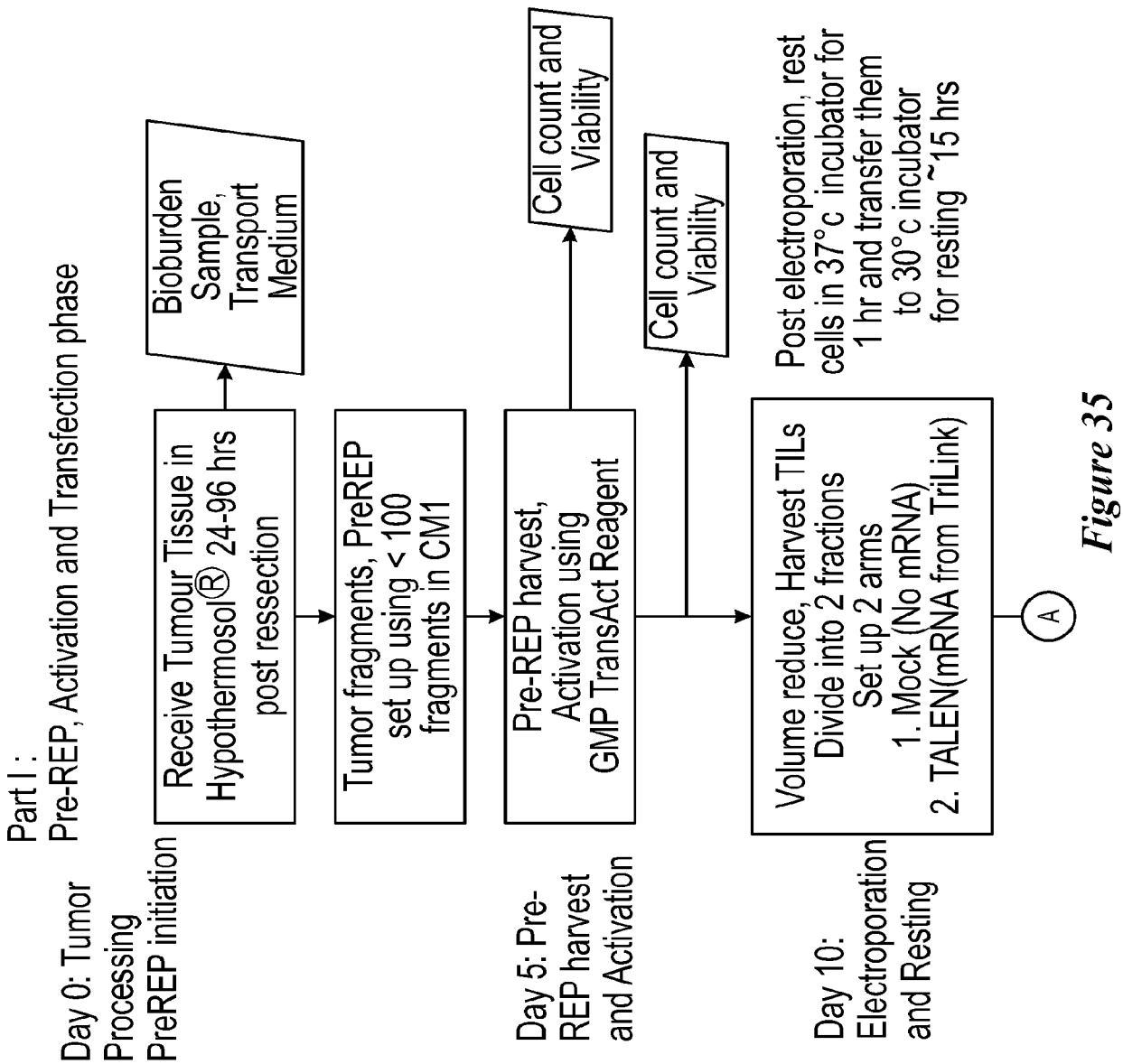
Part I :
Pre-REP, Activation and Transfection phase
Day 0: Tumor Processing PreREP initiation
Receive Tumour Tissue in Hypothermosol® 24-96 hrs post ressection
Bioburden Sample, Transport Medium
Tumor fragments, PreREP set up using < 100 fragments in CM1
Day 5: Pre-REP harvest and Activation
Pre-REP harvest, Activation using GMP TransAct Reagent
Cell count and Viability
Cell count and Viability
Day 10: Electroporation and Resting
Volume reduce, Harvest TILs
Divide into 2 fractions
Set up 2 arms
1. Mock (No mRNA)
2. TALEN(mRNA from TriLink)
A
Post electroporation, rest cells in 37°c incubator for 1 hr and transfer them to 30°c incubator for resting ~15 hrs

Figure 35

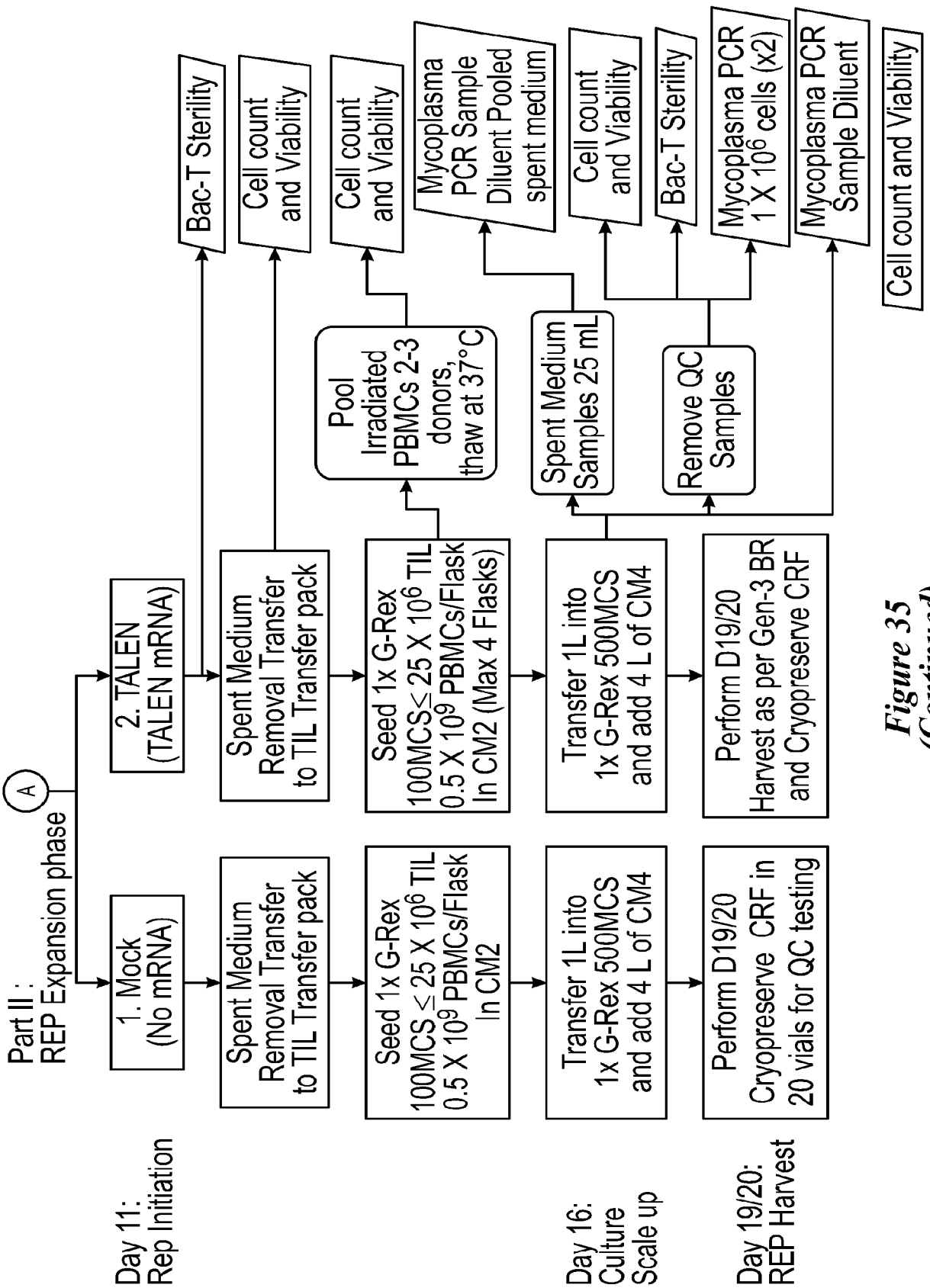

Figure 35 (Continued)

STEP A
Obtain Patient Tumor Sample (optionally can be frozen before Step B)

STEP B
First Expansion (physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing (culture TILs in medium comprising anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion (culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 36A

STEP A
Obtain Patient Tumor Sample (optionally can be frozen before Step B)

STEP B
First Expansion (physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 for about 3-9 days)

STEP C
Activation, Gene Editing (culture TILs in medium comprising anti-CD3 and anti-CD38 beads for about 1-7 days; electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion (culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 36B

STEP A
Obtain Patient Tumor Sample (optionally can be frozen before Step B)

STEP B
First Expansion (physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing (electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion (culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 5-15 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 36C

STEP A
Obtain Patient Tumor Sample (optionally can be frozen before Step B)

STEP B
First Expansion (physical fragmentation and/or enzymatic digest; culture TILs in medium comprising IL-2 and OKT-3 for about 3-9 days)

STEP C
Gene Editing (electroporate TILs with TALEN mRNAs; rest for about 1 day)

STEP D
Rapid Second Expansion (culture TILs in medium comprising APCs, OKT-3, and IL-2 for about 1-7 days; scale up and add culture in medium comprising IL-2 for about 3-6 days)

STEP E
Harvest TILs from Step D

STEP F
Final Formulation and/or Transfer to Infusion Bag (optionally cryopreserve)

Figure 36D

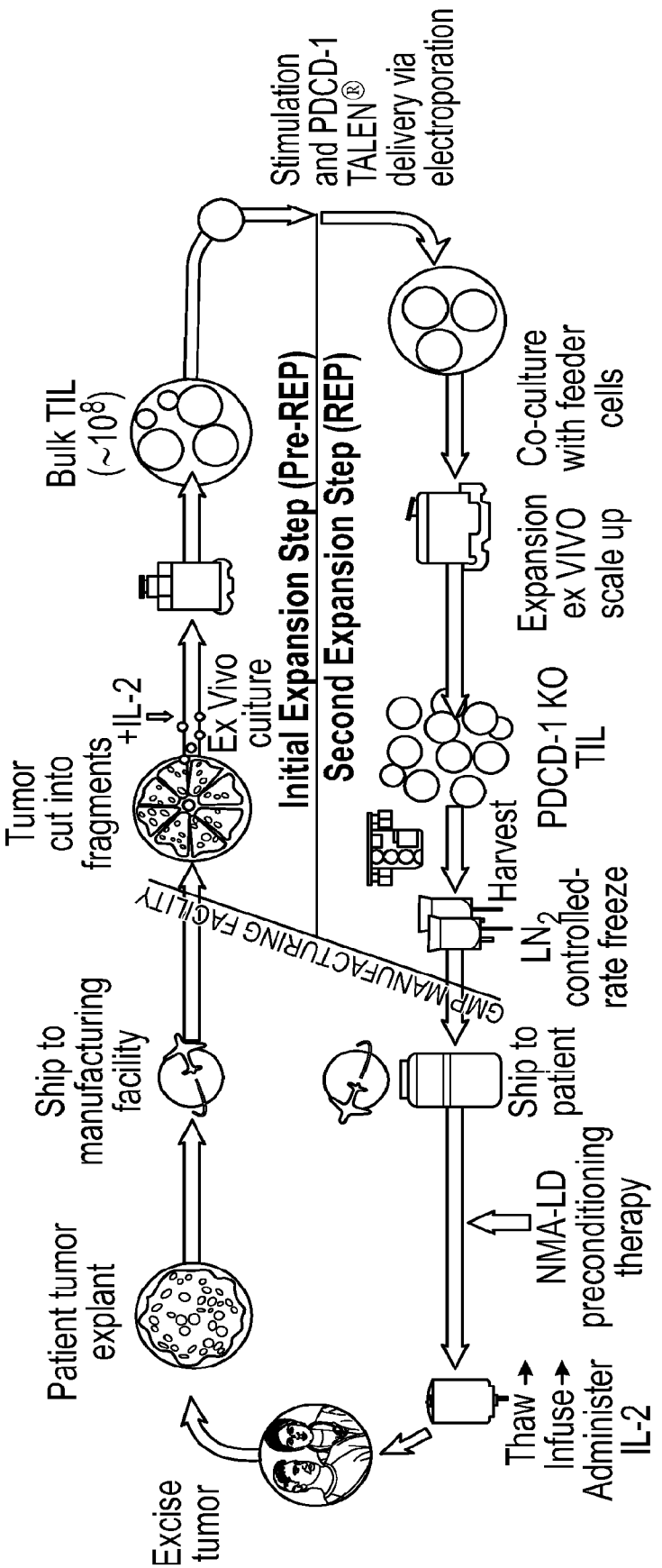
Excise tumor
Patient tumor explant
Ship to manufacturing facility
Tumor cut into fragments
+IL-2
Ex Vivo culture
Bulk TIL (~10^8)
Stimulation and PDCD-1 TALEN® delivery via electroporation
Initial Expansion Step (Pre-REP)
Second Expansion Step (REP)
GMP MANUFACTURING FACILITY
Co-culture with feeder cells
Expansion ex VIVO scale up
Harvest
PDCD-1 KO TIL
LN2 controlled-rate freeze
Ship to patient
NMA-LD preconditioning therapy
Thaw → Infuse → Administer IL-2

Figure 37

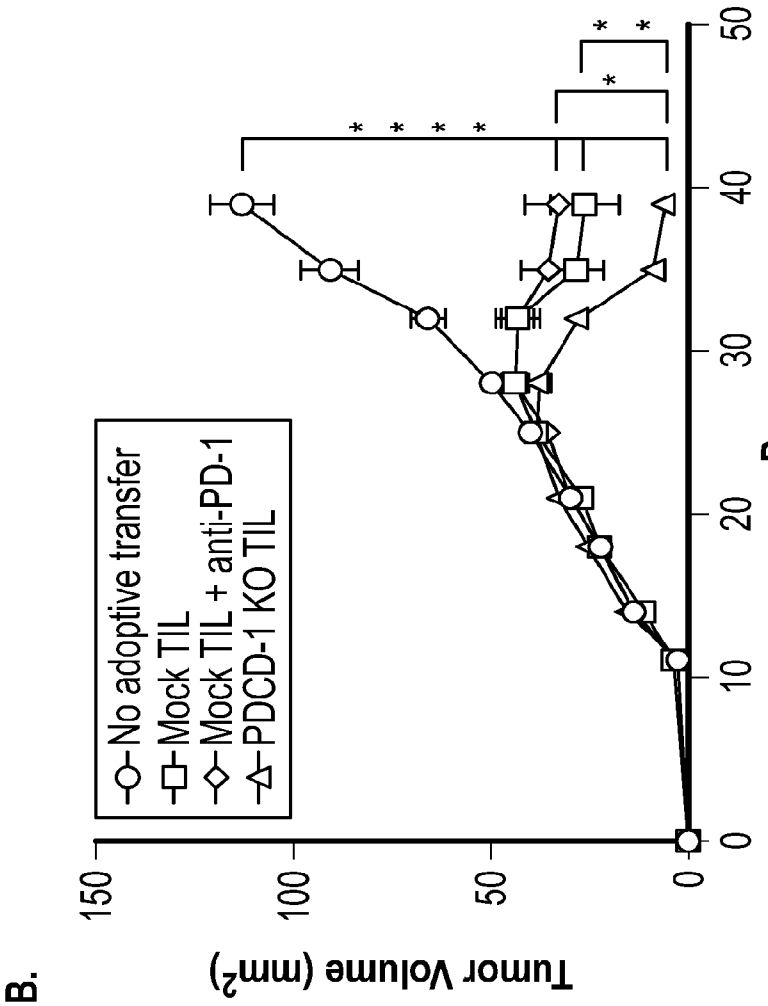

Figure 38B

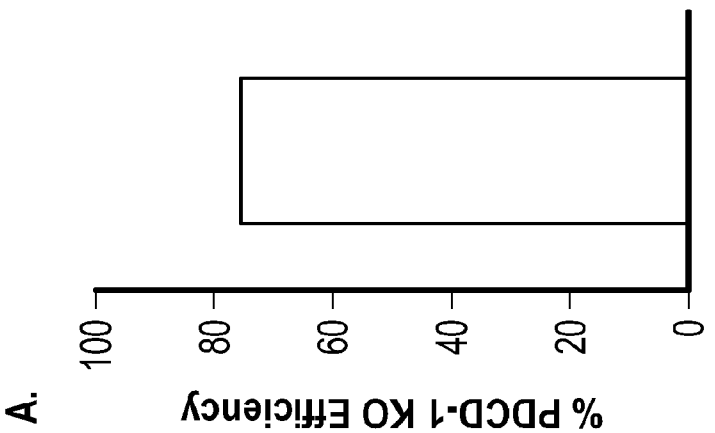

Figure 38A

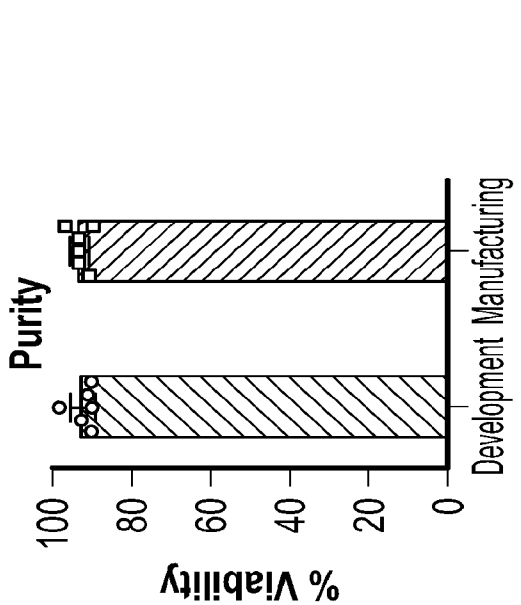

Figure 39A

Figure 39B

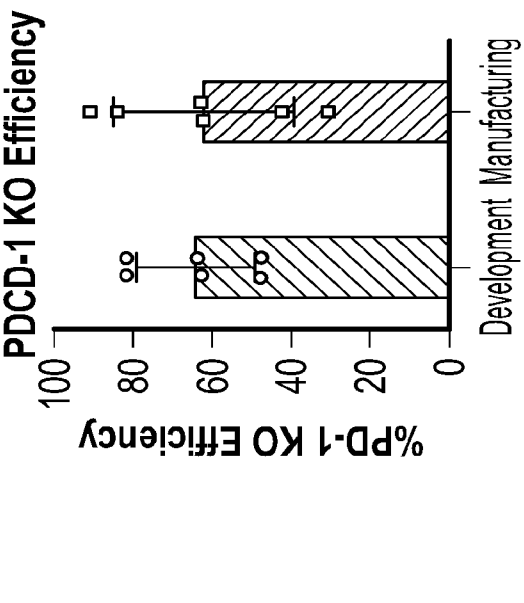

Figure 39E

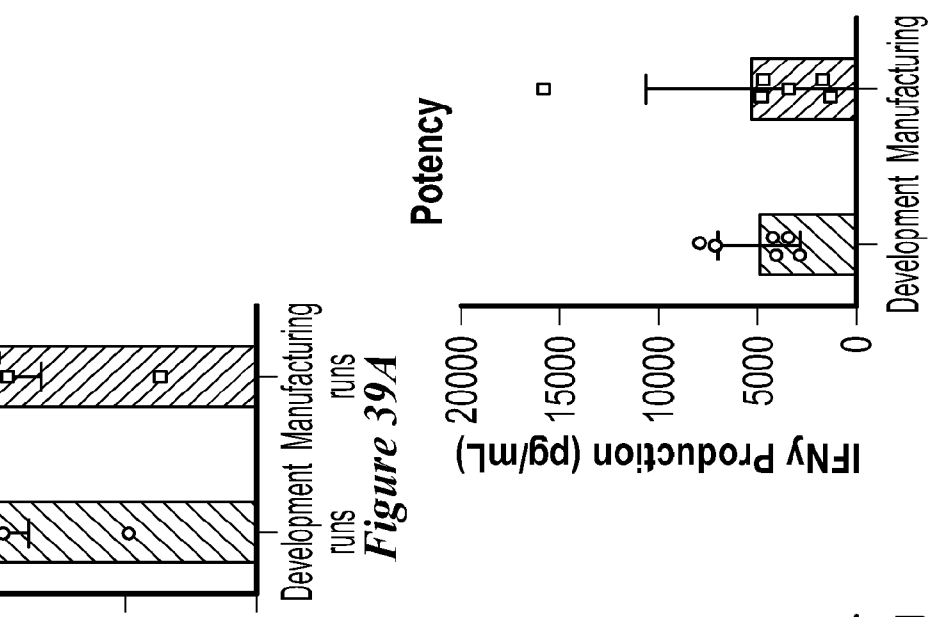

Figure 39D

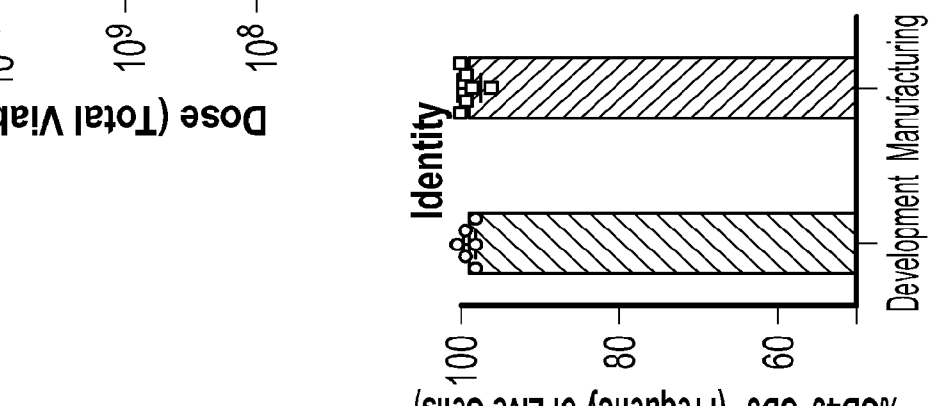

Figure 39C

Values are displayed as mean ±SD

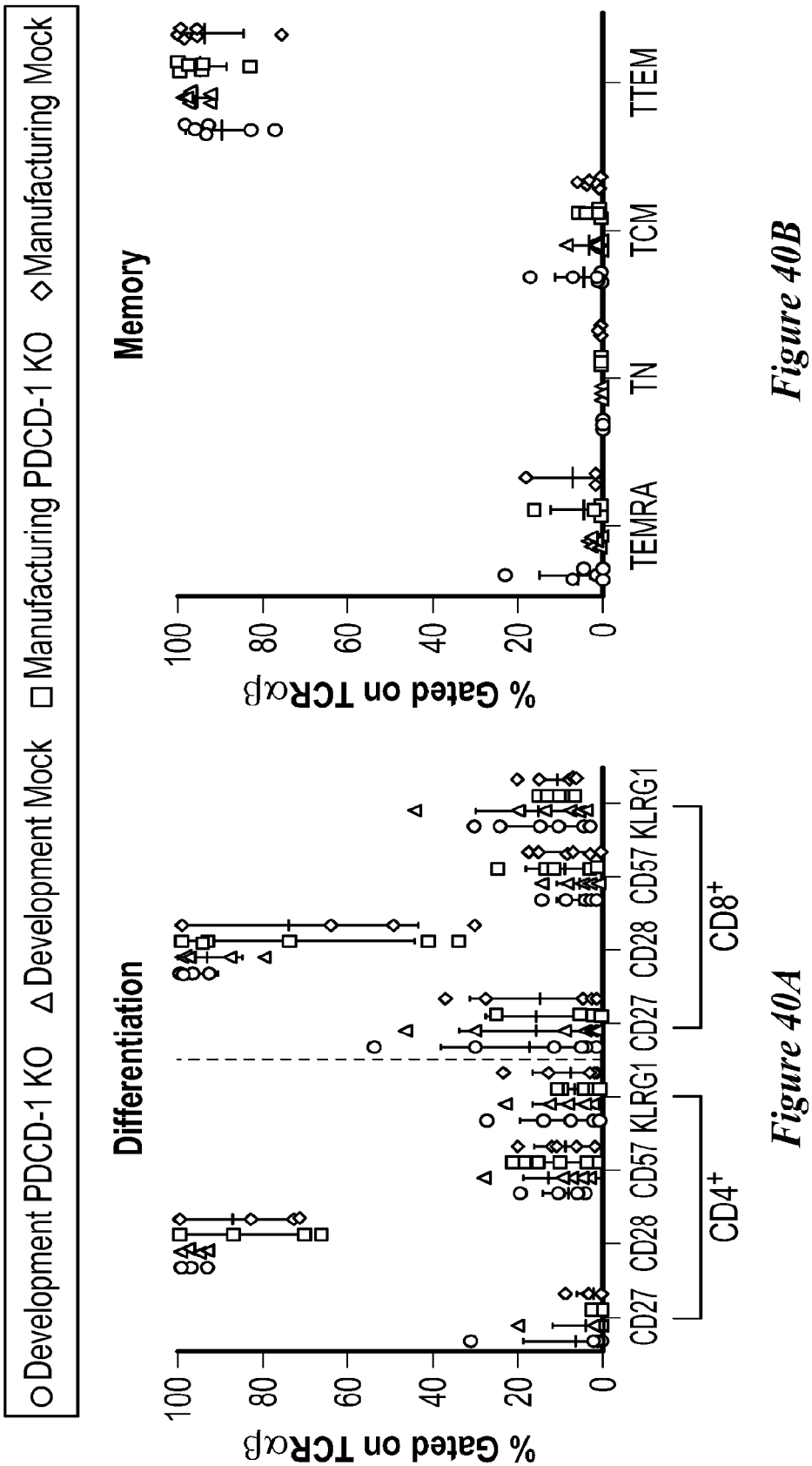

Figure 40A

Figure 40B

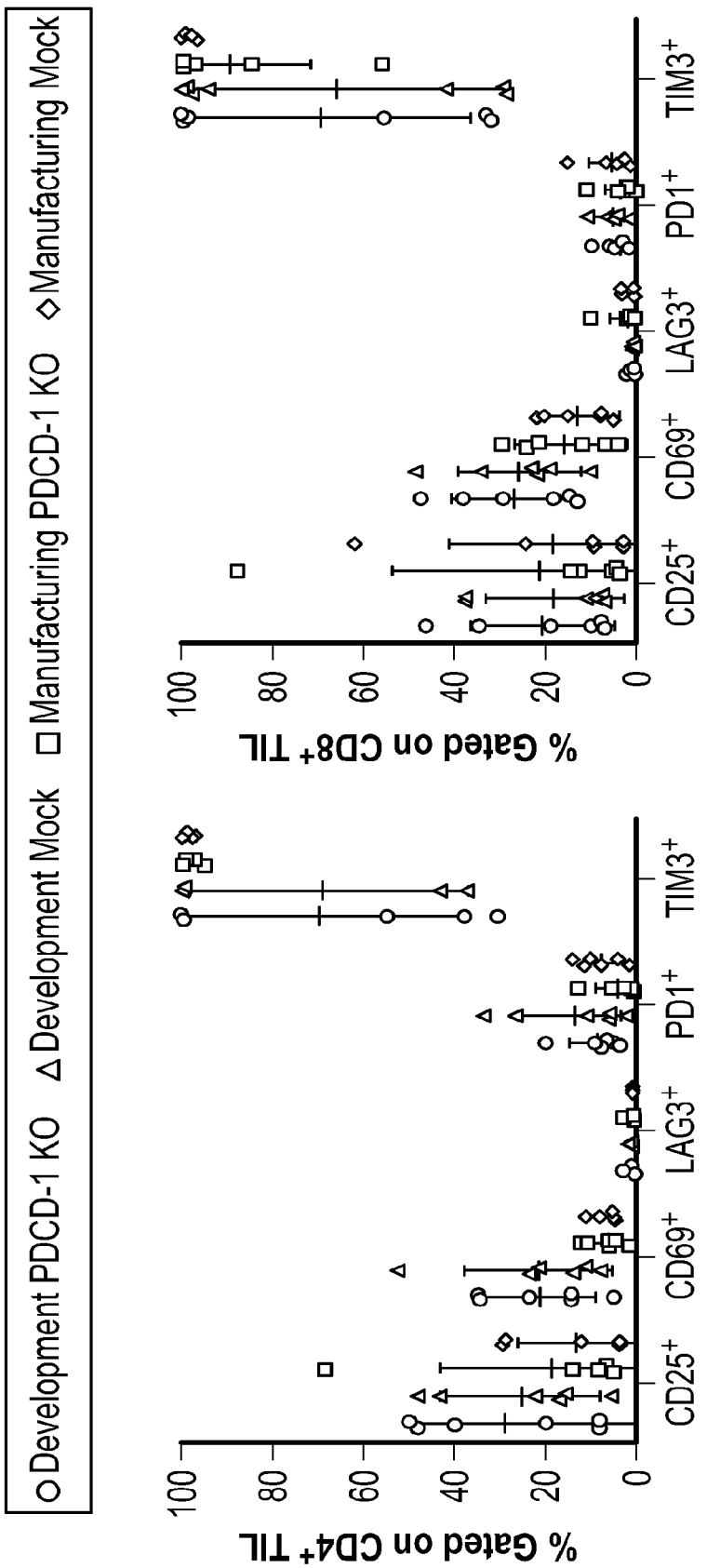

Figure 41A

Figure 41B

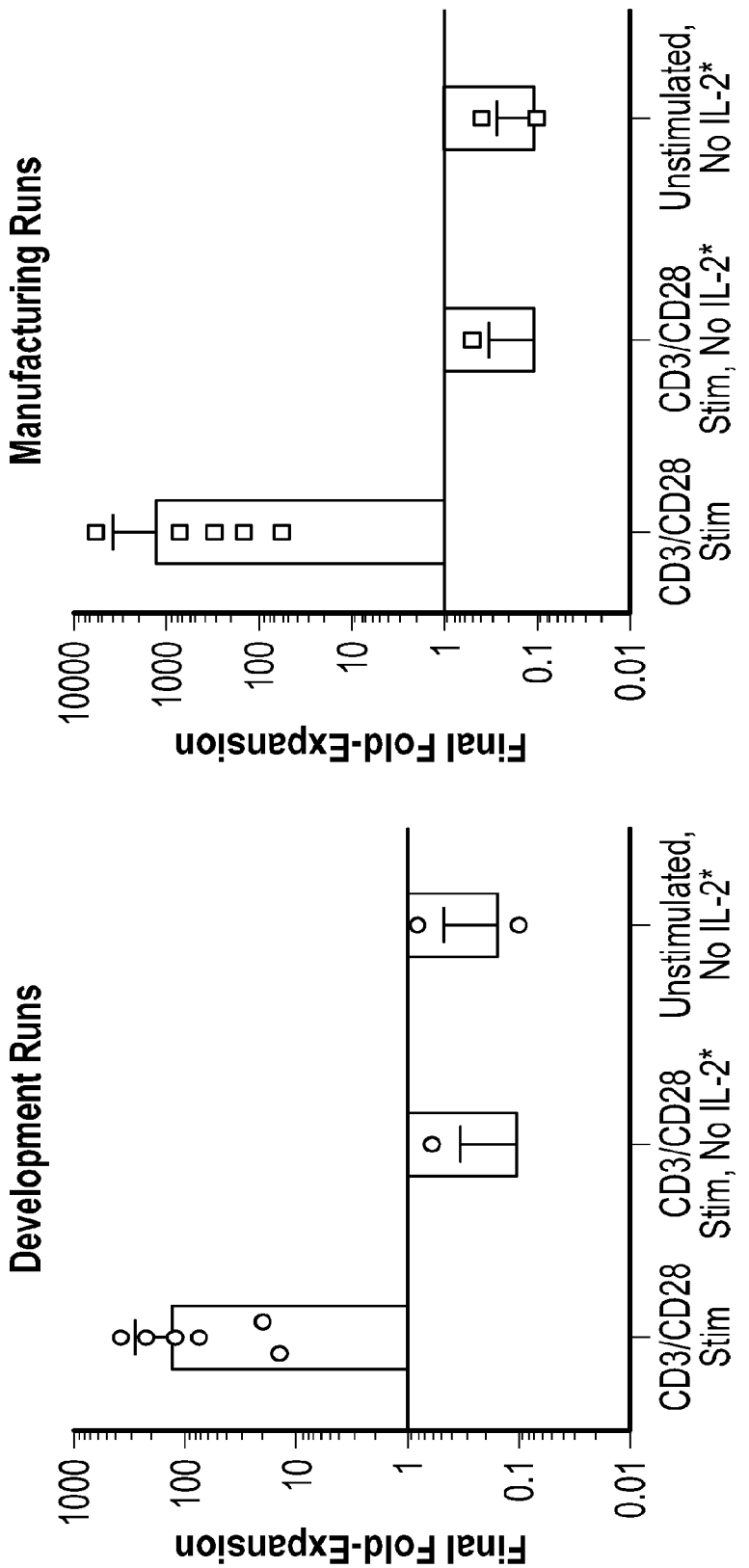

Figure 42A

Figure 42B

*Cell count data < lower limit of quantitation on the NC200 cell count.. Green circles and squares represent individual samples within the development and manufacturing runs, respectively, and values are displayed as mean + SD.

G-Banding Results		
Sample ID	Mock	PDCD-1 KO
1	Normal female: 46,[a]XX[b] [20][c]	Normal female: 46,[a]XX[b] [20][c]
2	Normal male: 46,[a]XX[b] [20][c]	Normal male: 46,[a]XX[b] [20][c]
3	Normal female: 46,[a]XX[b] [20][c]	Normal female: 46,[a]XX[b] [20][c]
4	Normal male: 46,[a]XX[b] [20][c]	Normal male: 46,[a]XX[b] [20][c]
5	Normal male: 46,[a]XX[b] [20][c]	Normal male: 46,[a]XX[b] [20][c]
6	Normal female: 46,[a]XX[b] [20][c]	Normal female: 46,[a]XX[b] [20][c]

[a]Number of chromosomes.
[b]Sex complement.
[c]Number of analyzed metaphase cells.

Figure 43

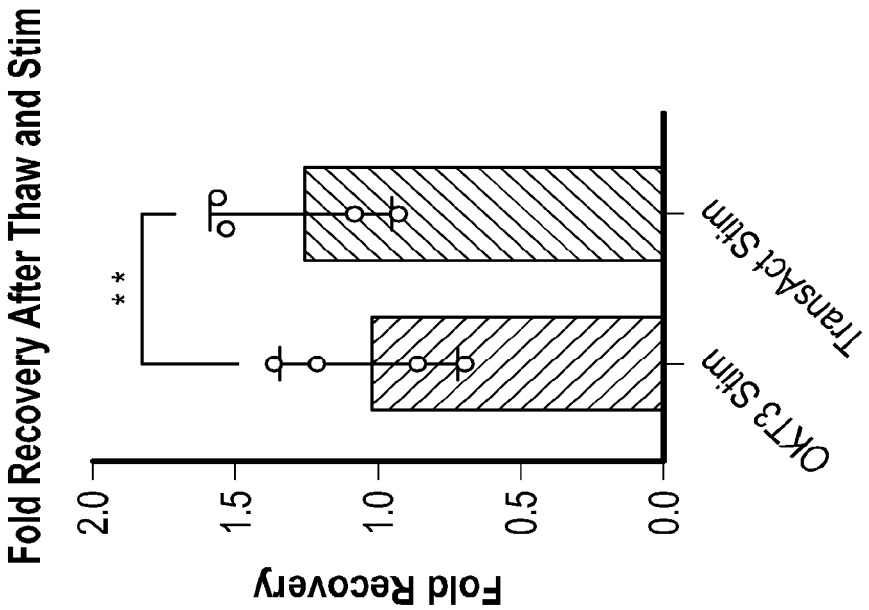

Figure 44B

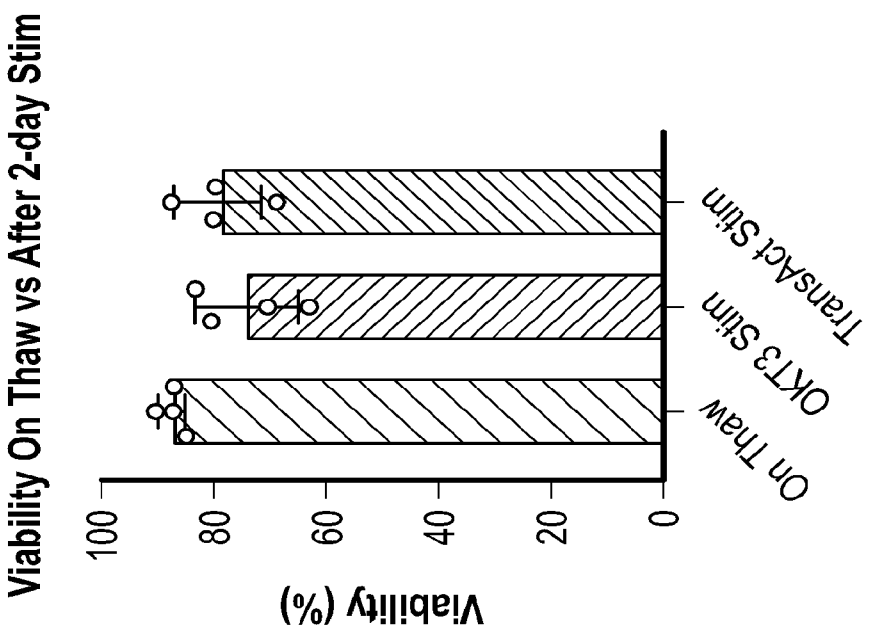

Figure 44A

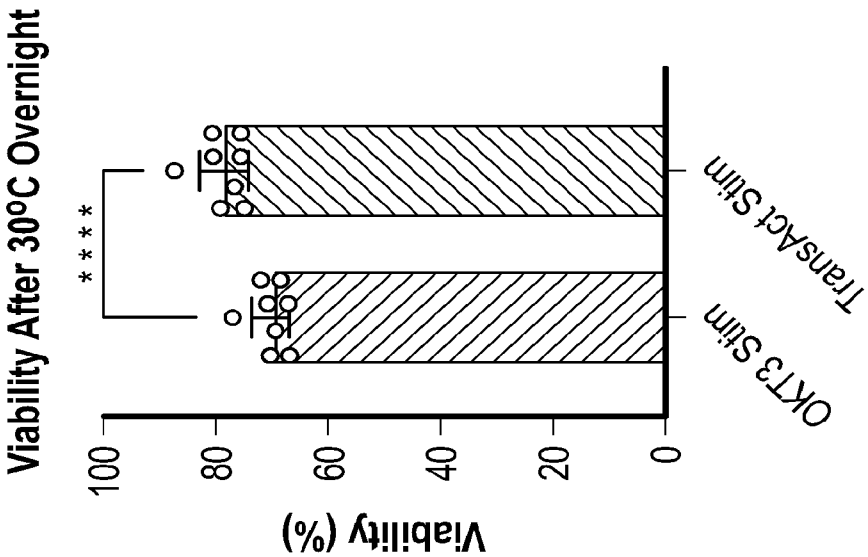

Figure 45B

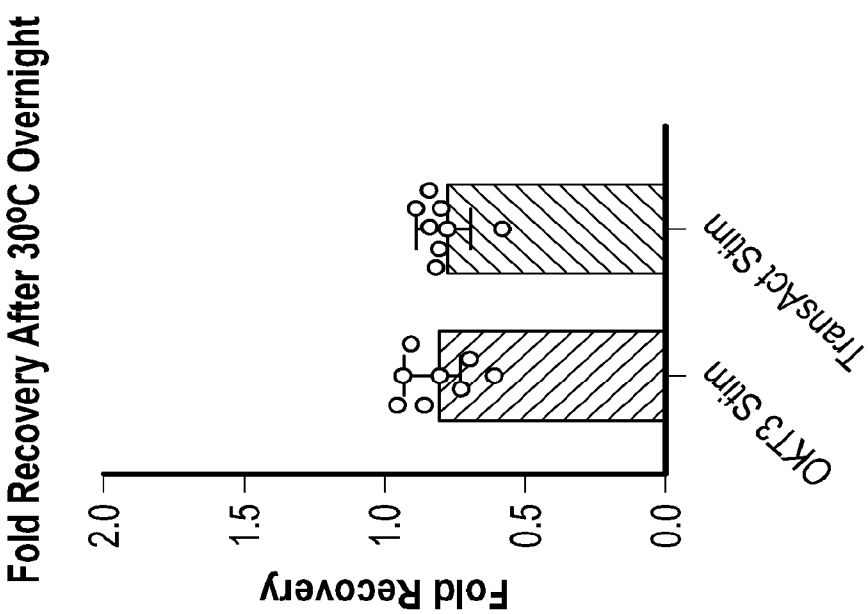

Figure 45A

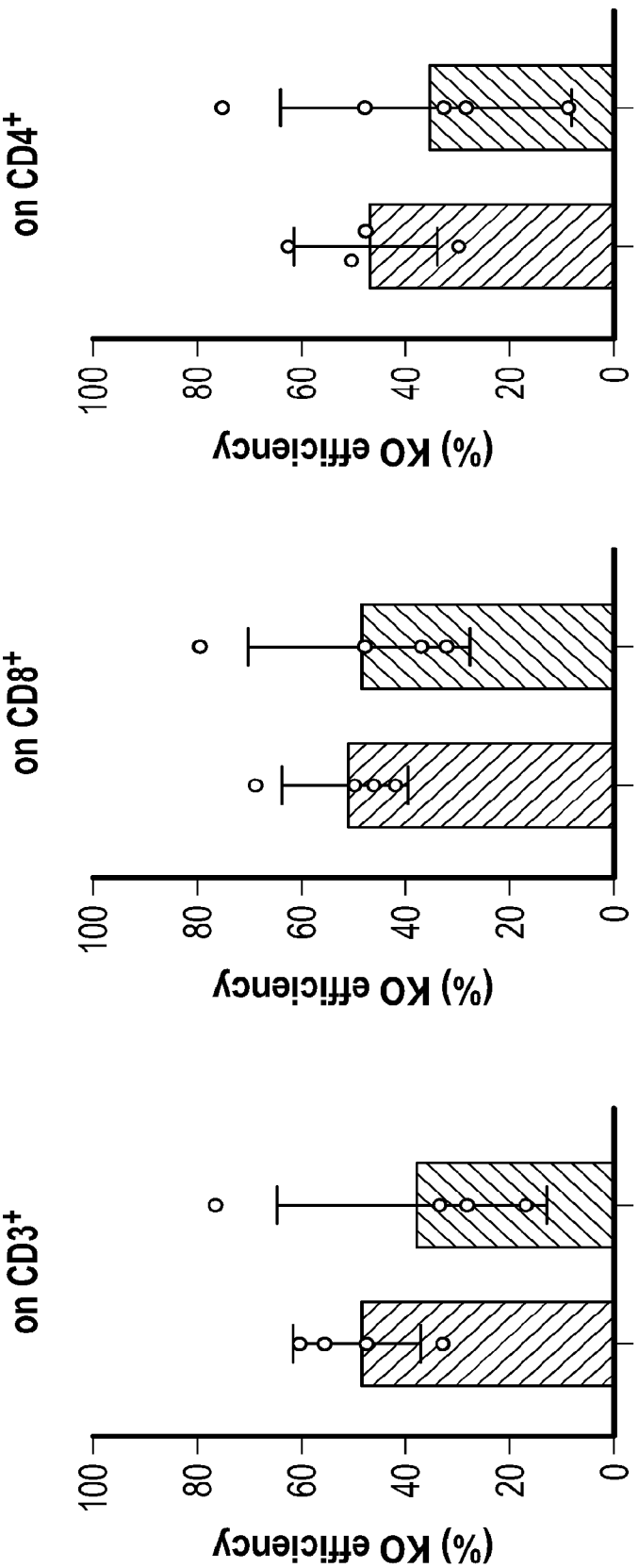

Figure 46A

Figure 46B

Figure 46C

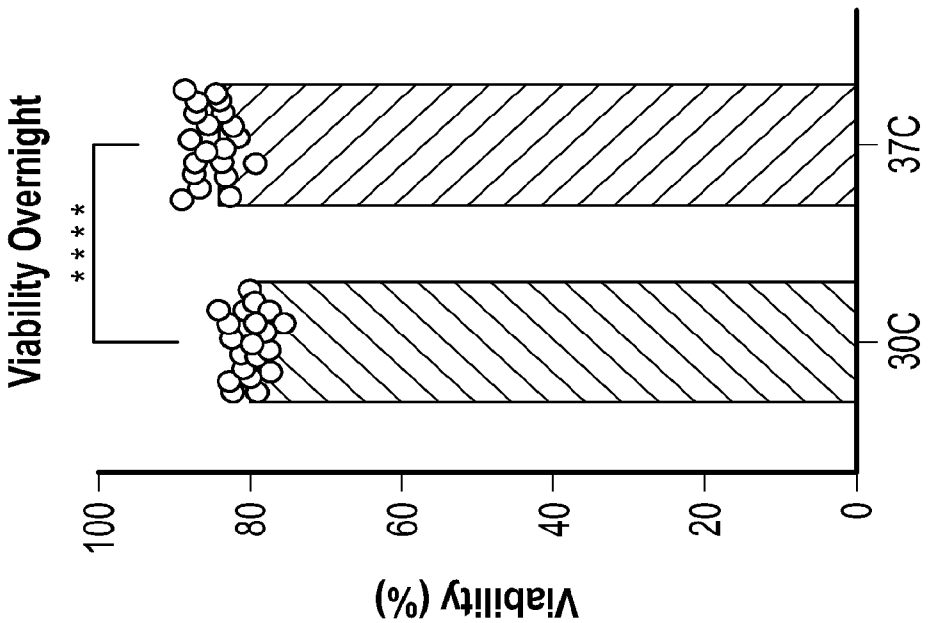

Figure 47B

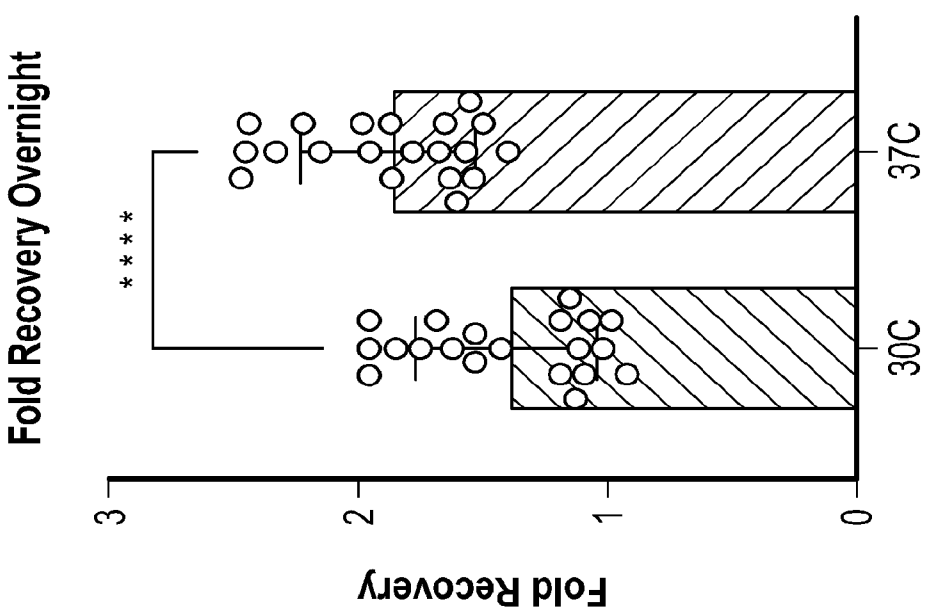

Figure 47A

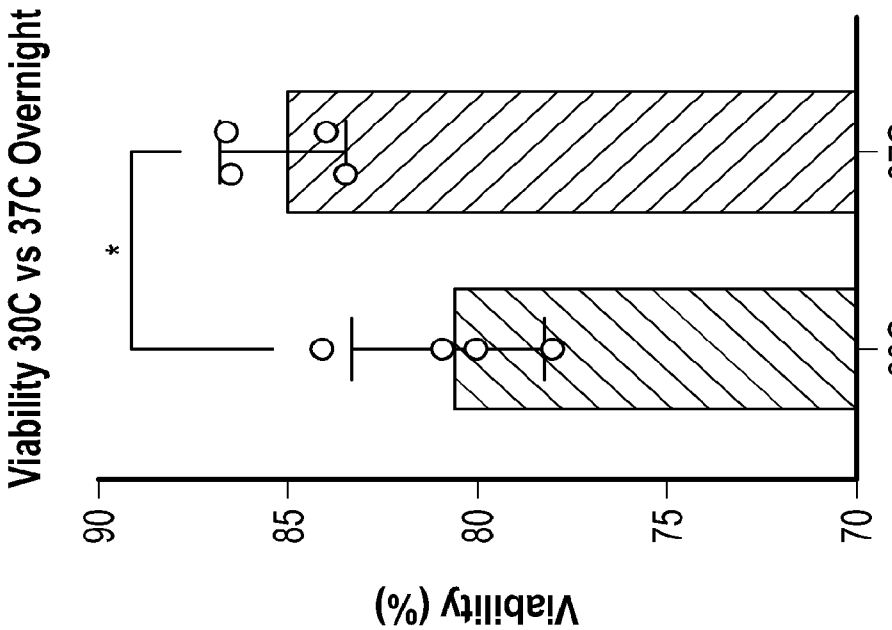

Figure 48B

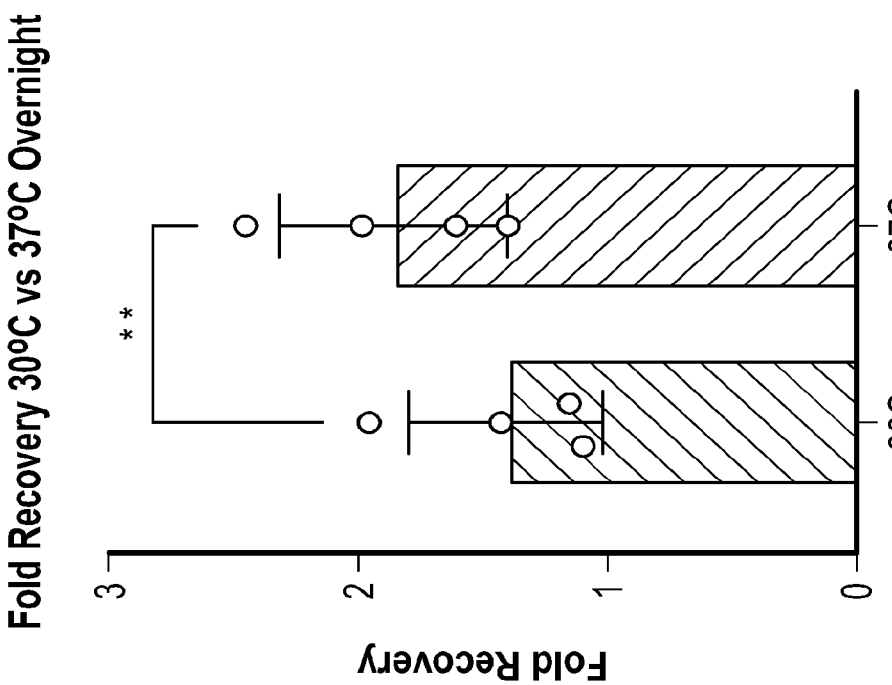

Figure 48A

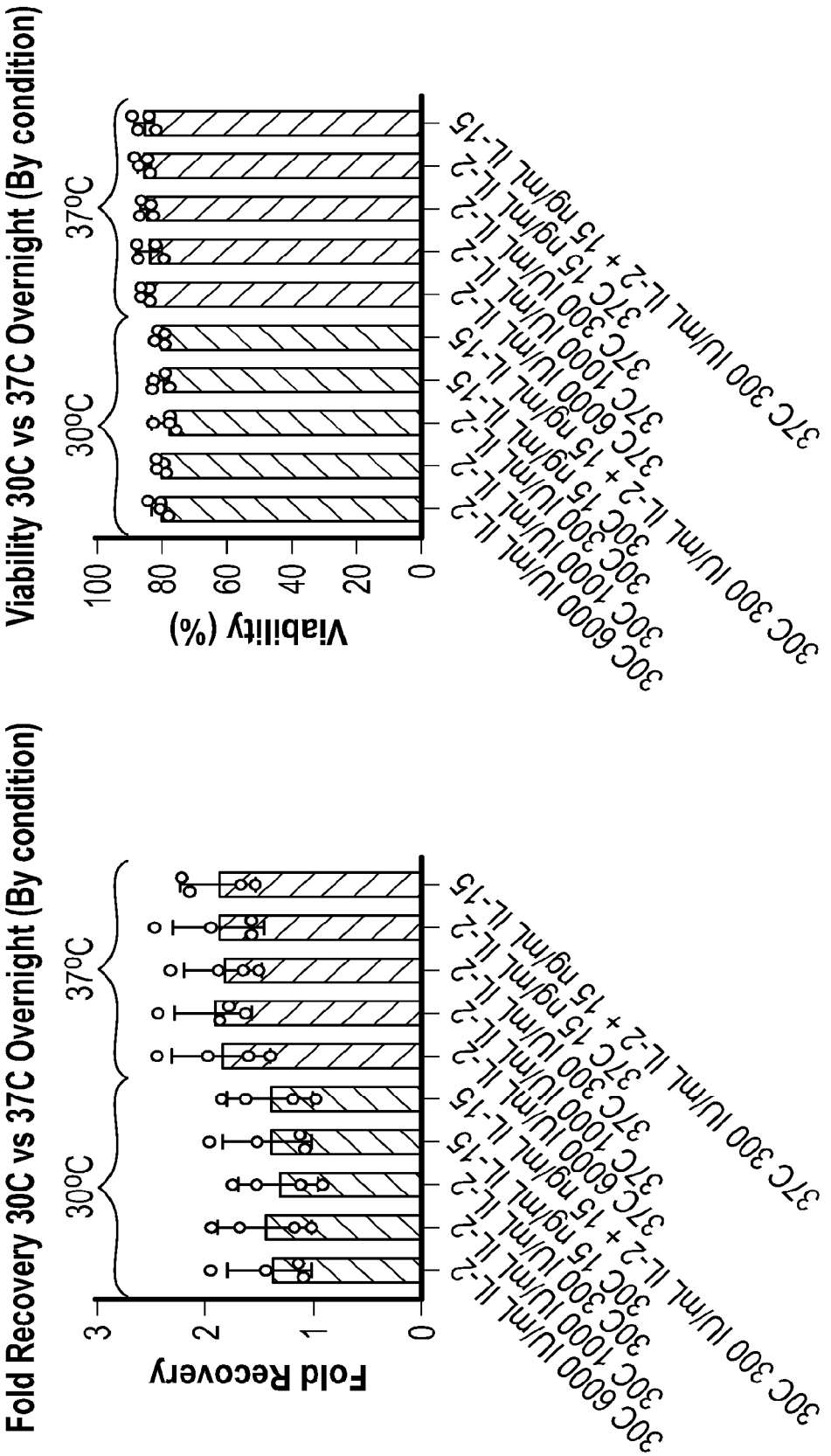

Figure 49A

Figure 49B

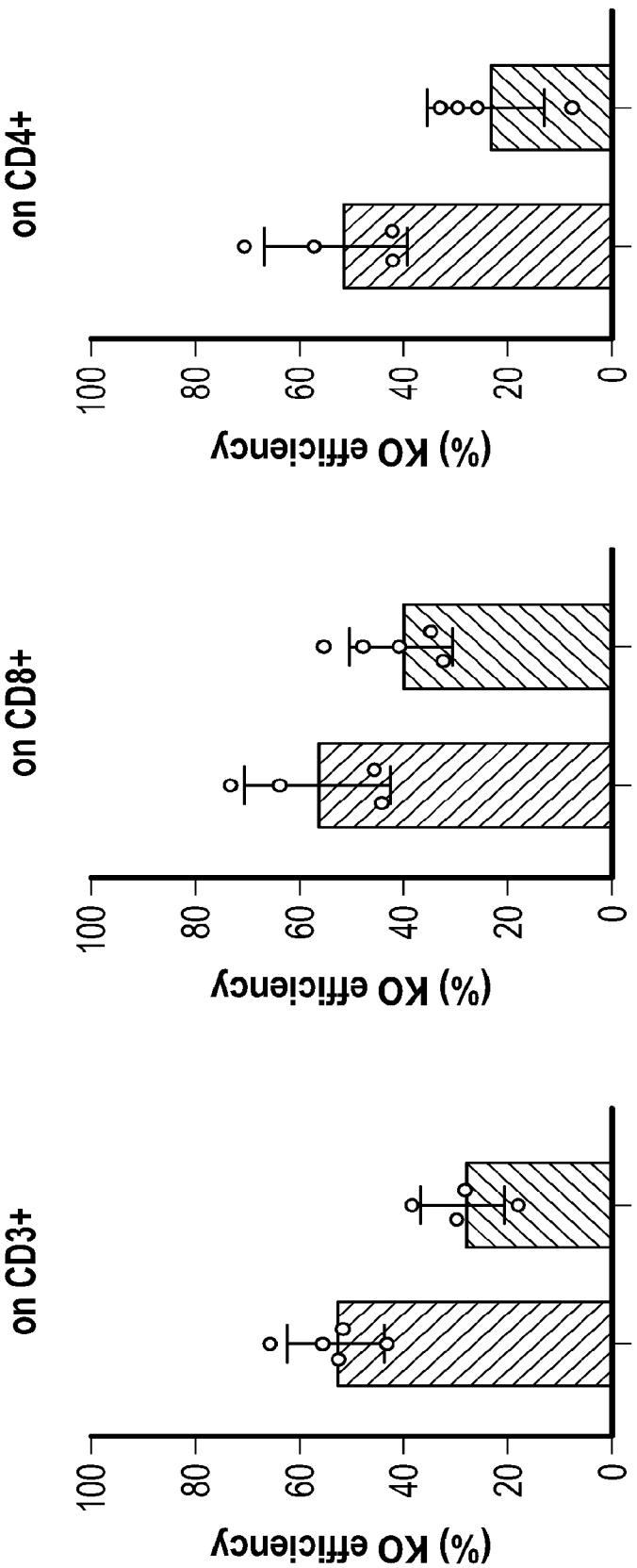

Figure 50A

Figure 50B

Figure 50C

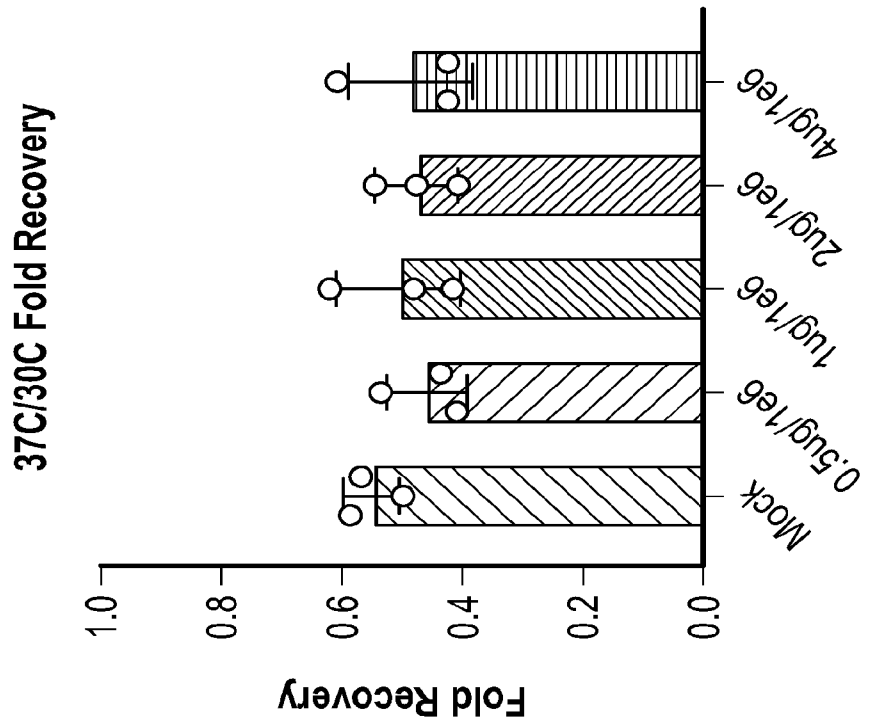

Figure 52

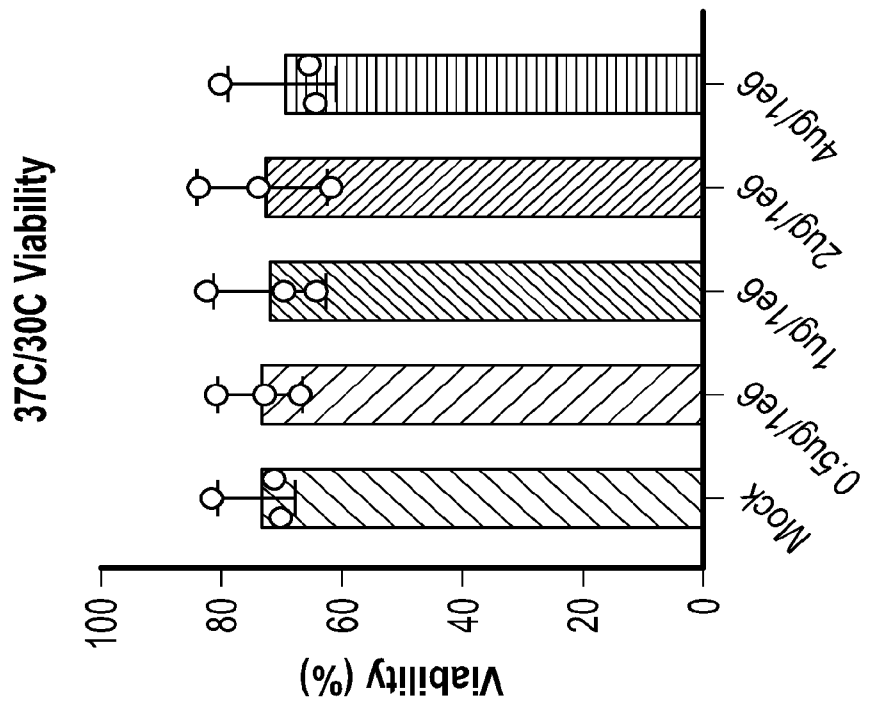

Figure 51

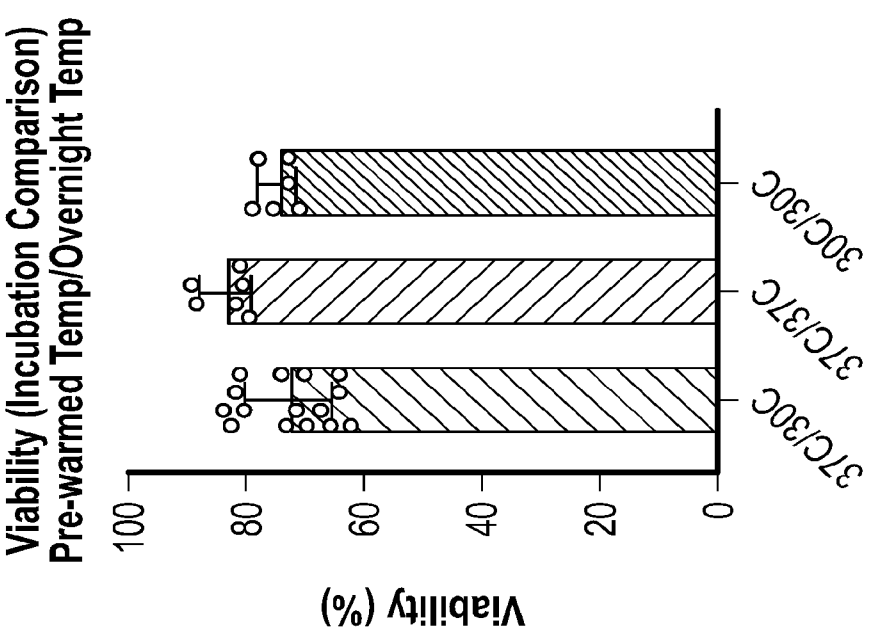

Figure 53B

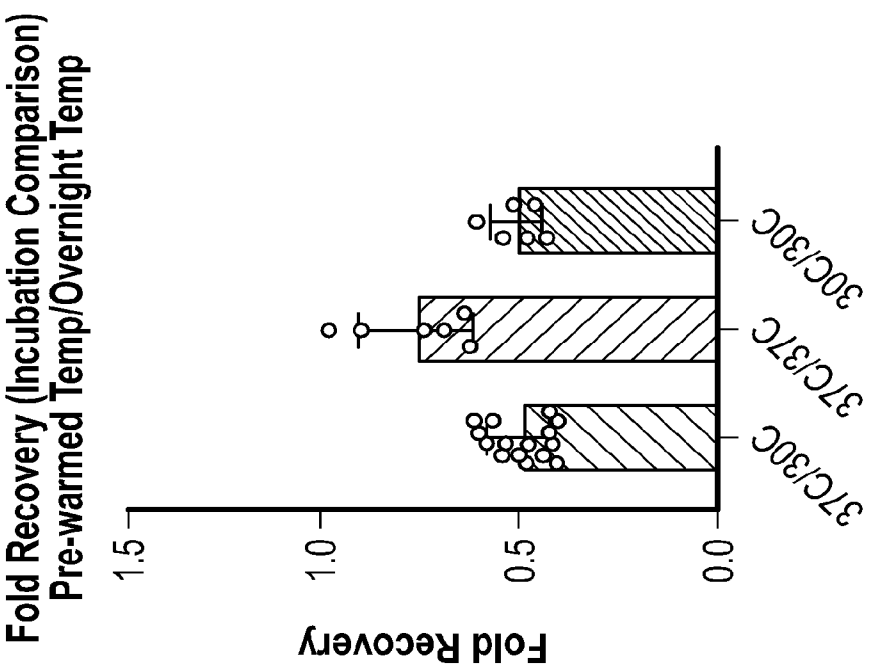

Figure 53A

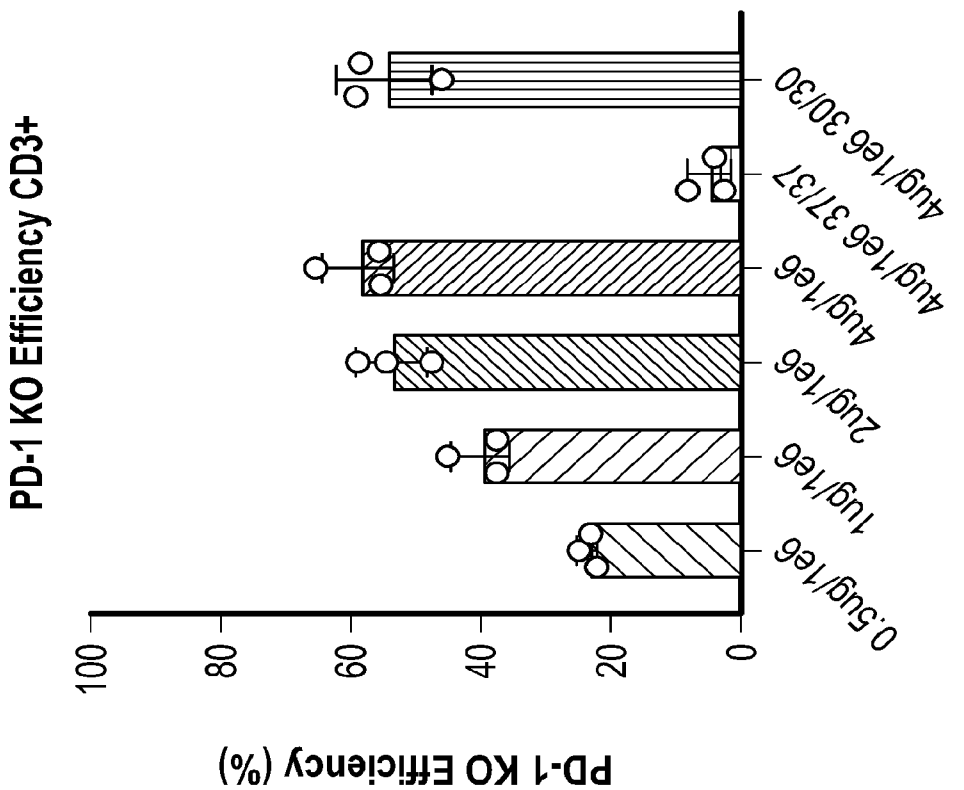
PD-1 KO Efficiency CD3+
PD-1 KO Efficiency (%)
100
80
60
40
20
0
0.5ug/1e6
1ug/1e6
2ug/1e6
4ug/1e6
4ug/1e6 37/37
4ug/1e6 30/30

Figure 54A

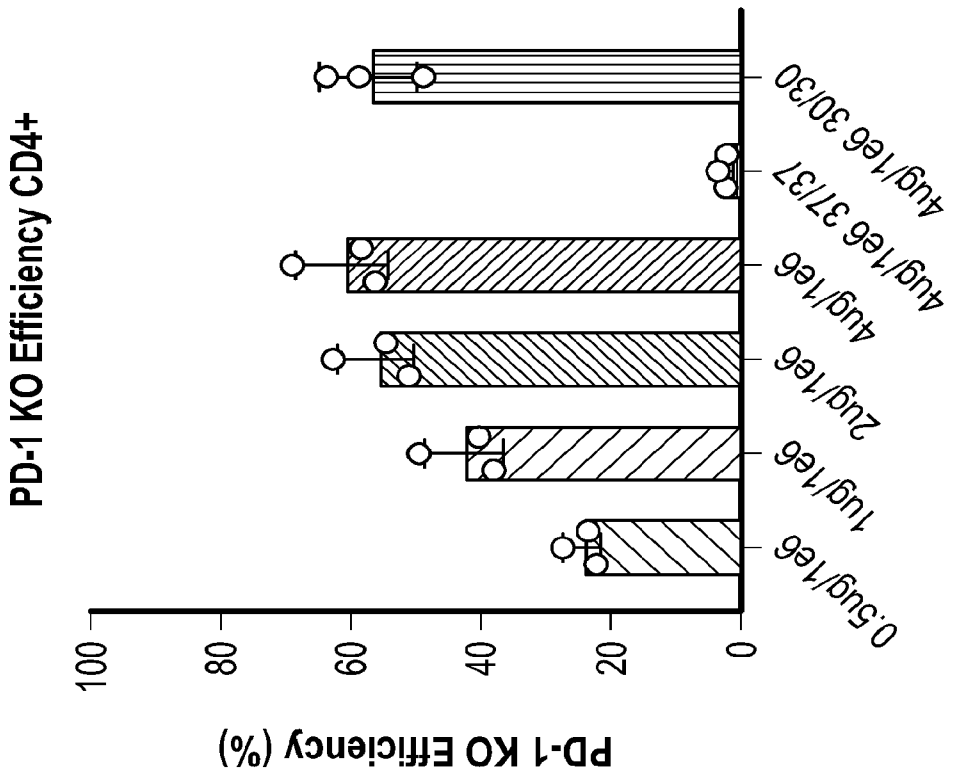

Figure 54C

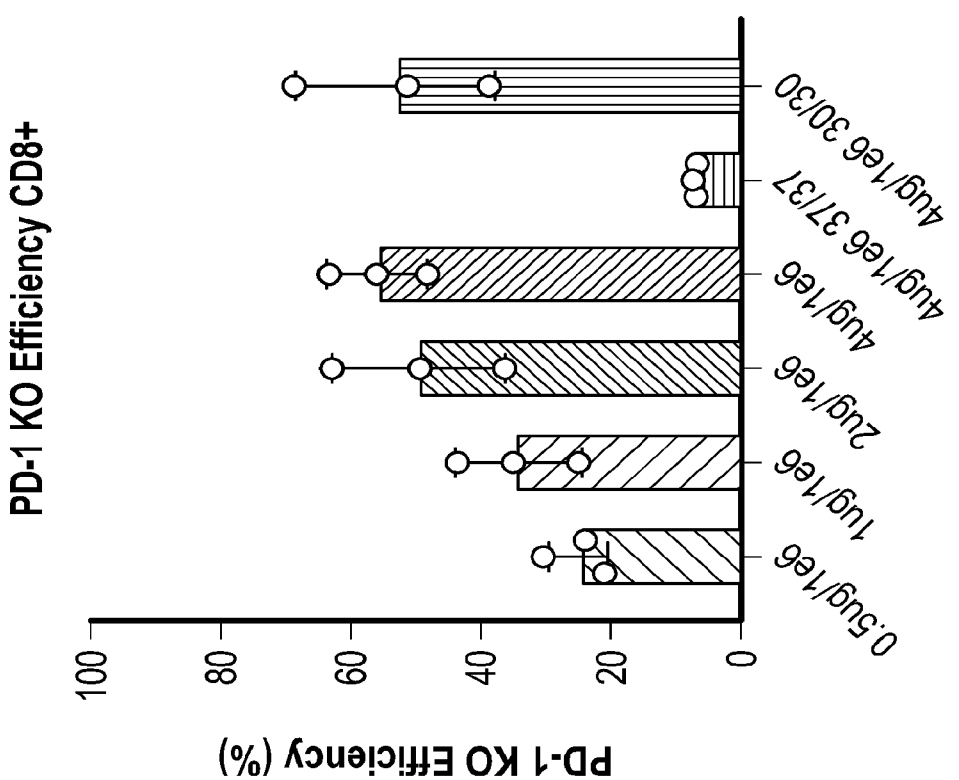

Figure 54B

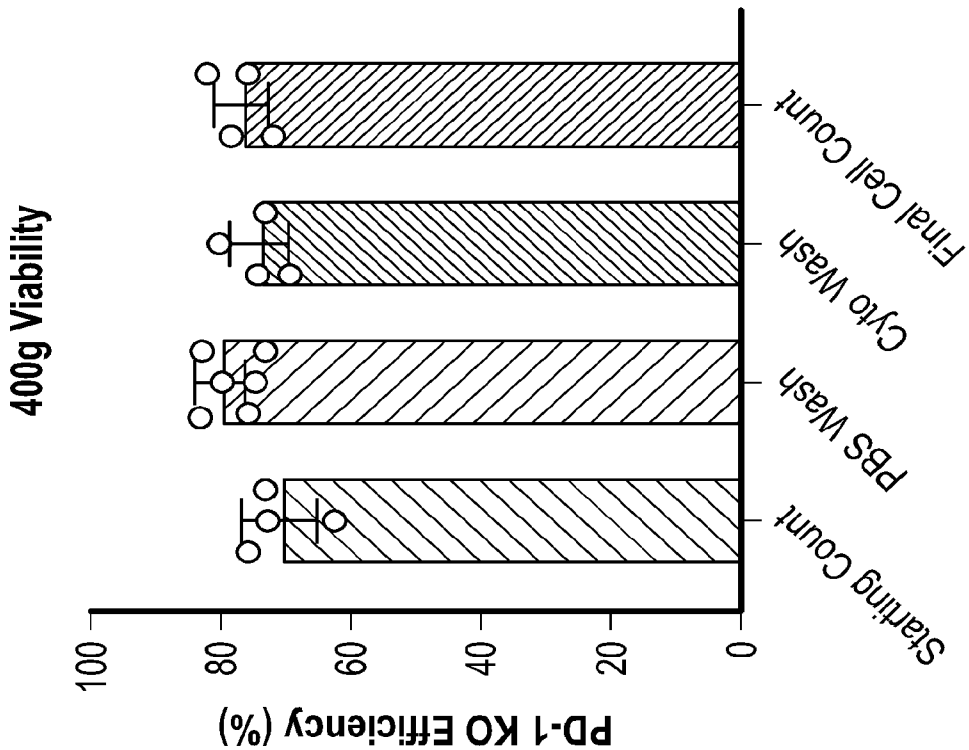

Figure 55B

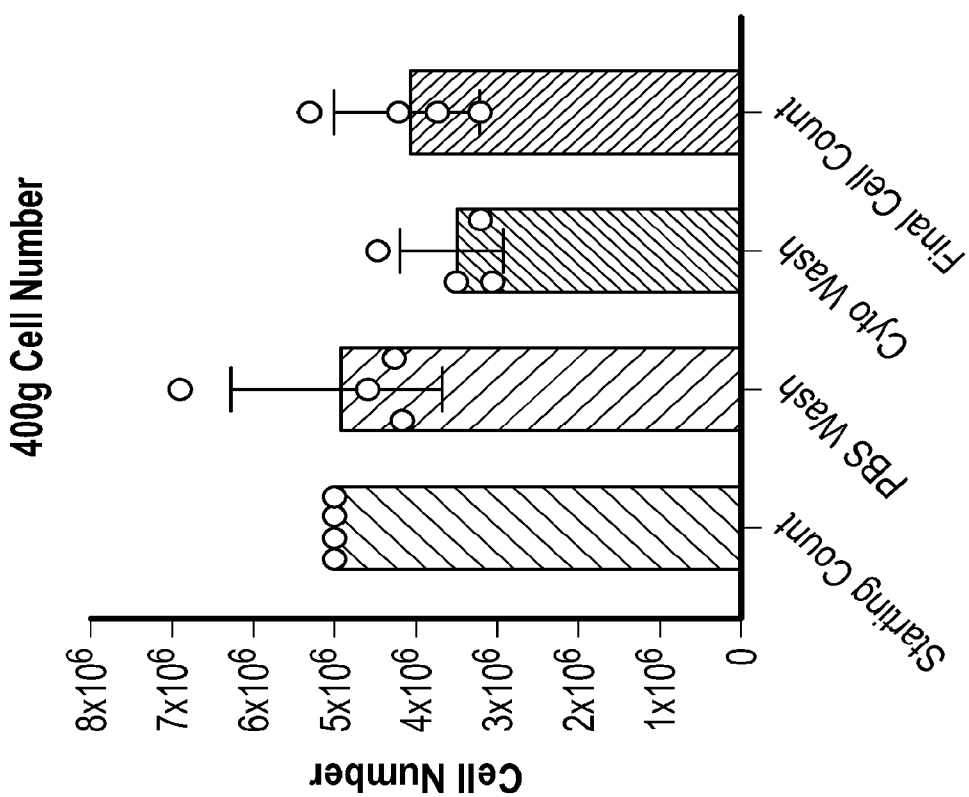

Figure 55A

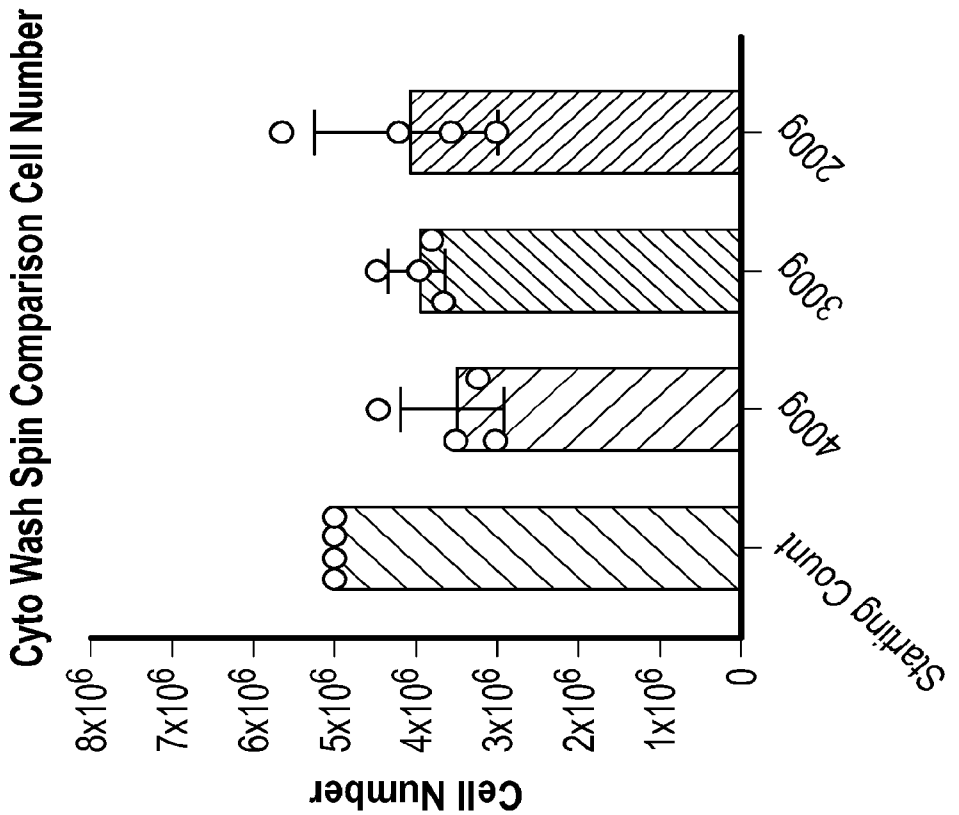

Figure 56B

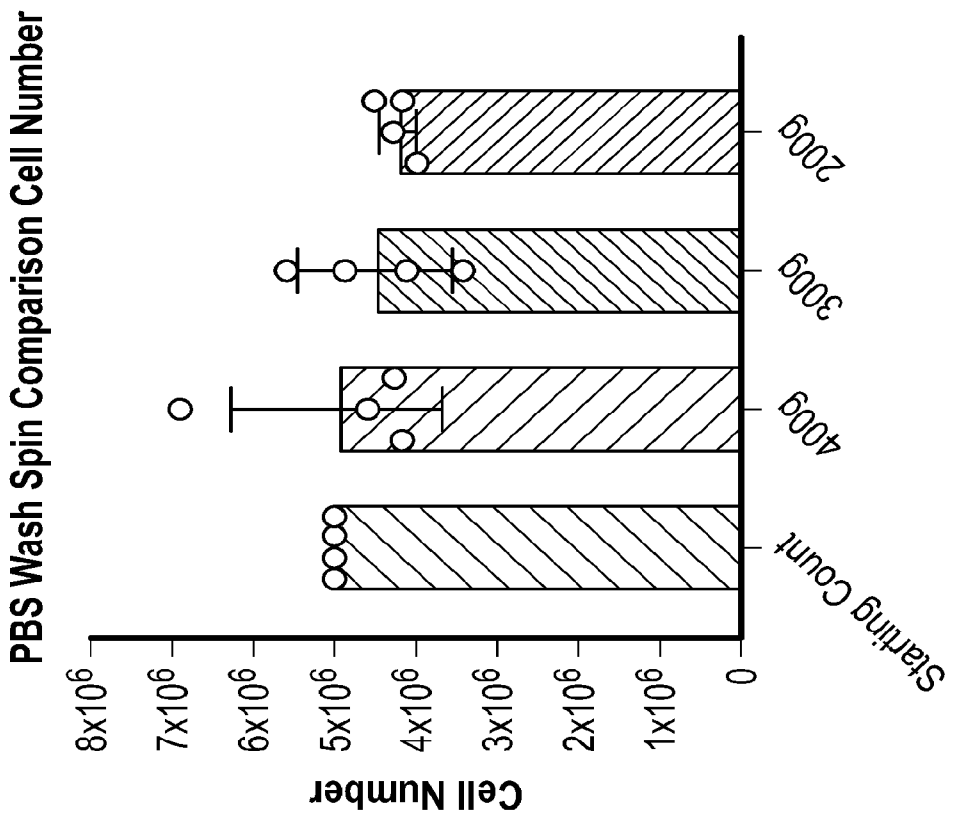

Figure 56A

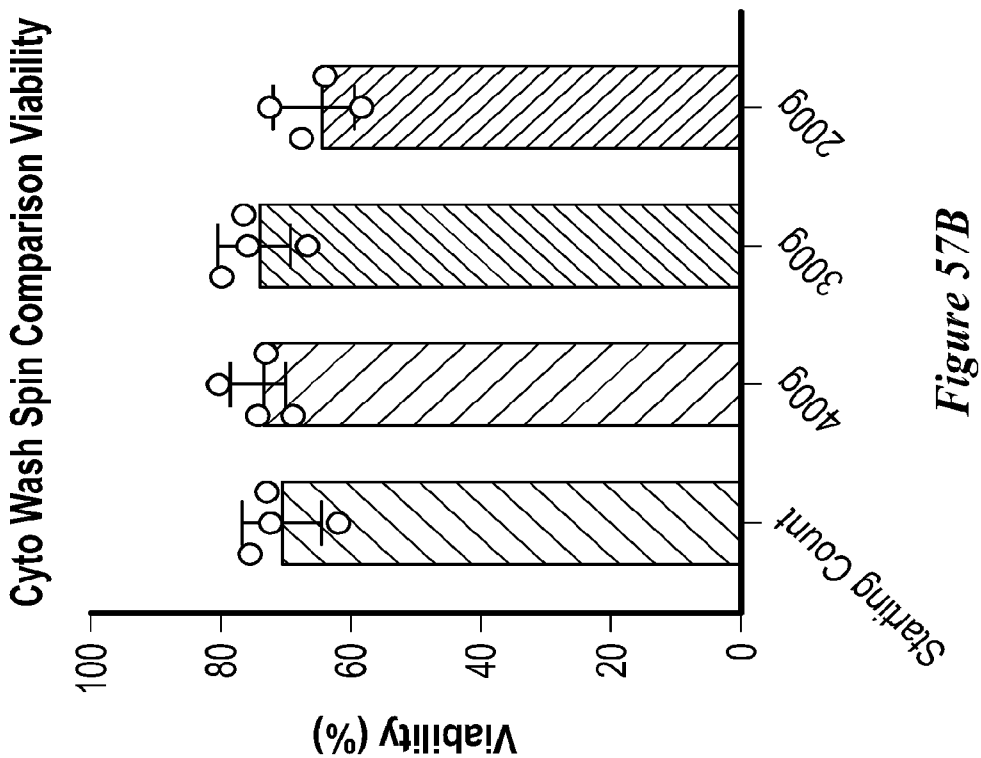

Figure 57B

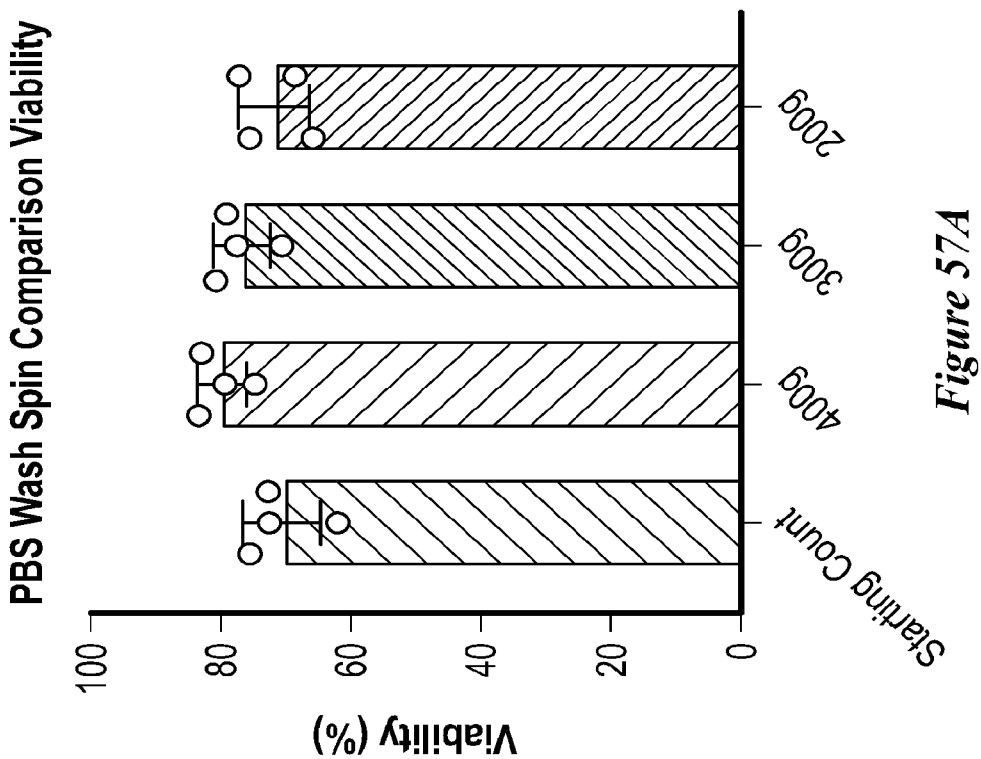

Figure 57A

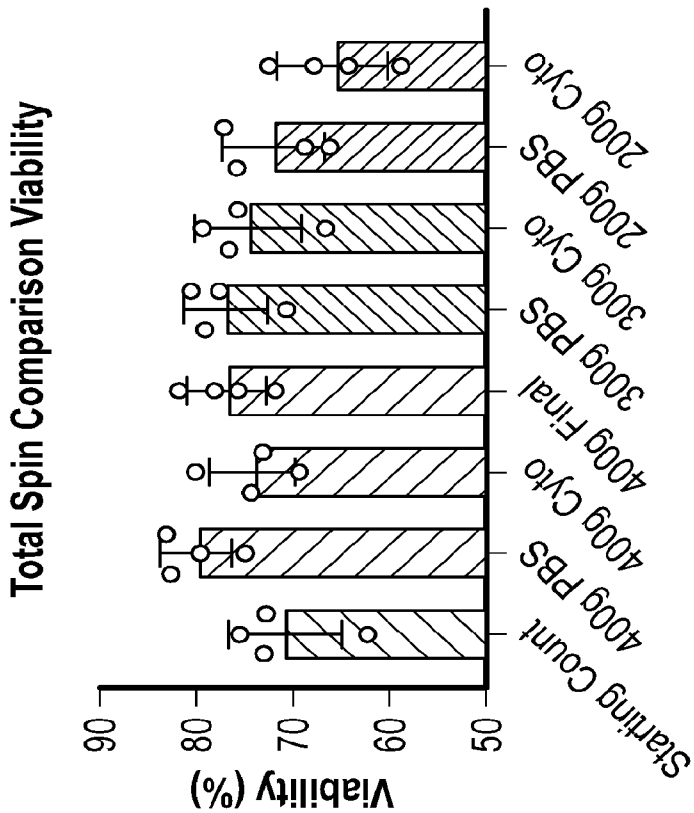

Figure 58B

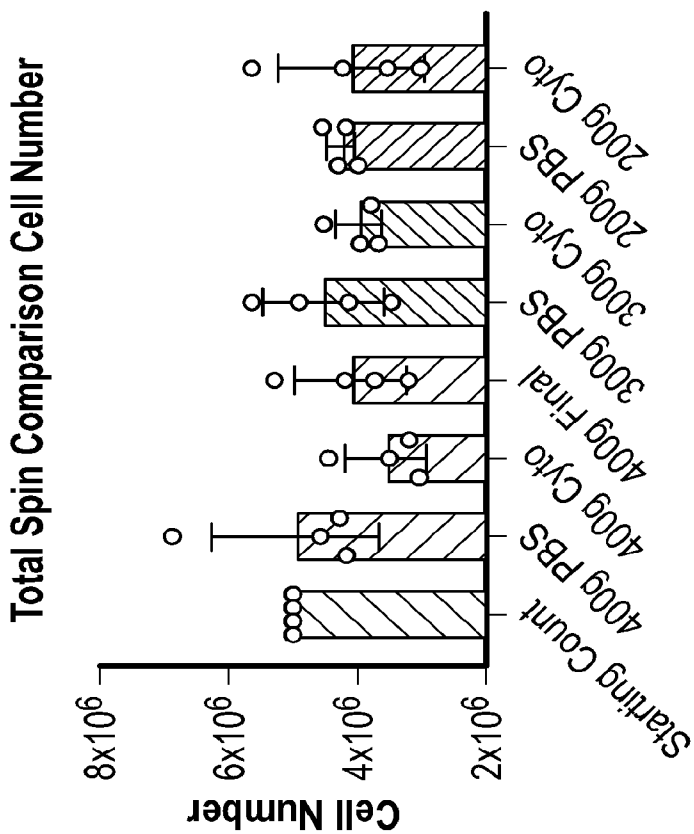

Figure 58A

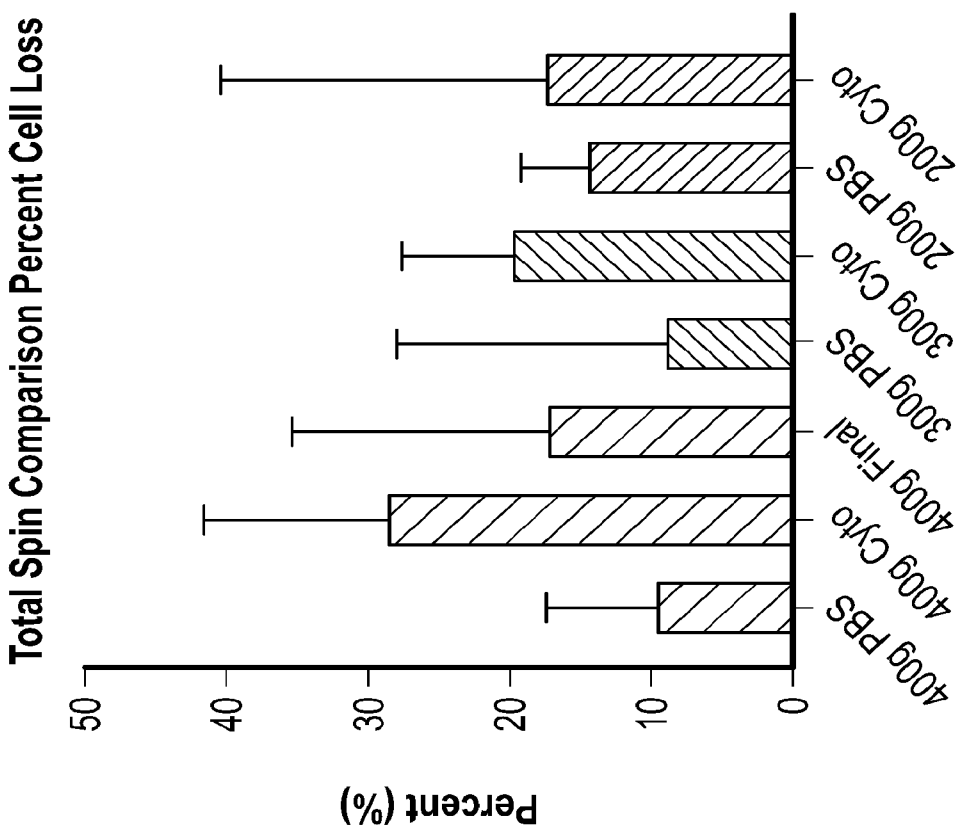
Total Spin Comparison Percent Cell Loss
Percent (%)
0
10
20
30
40
50
400g PBS
400g Cyto
400g Final
300g PBS
300g Cyto
200g PBS
200g Cyto

Figure 59

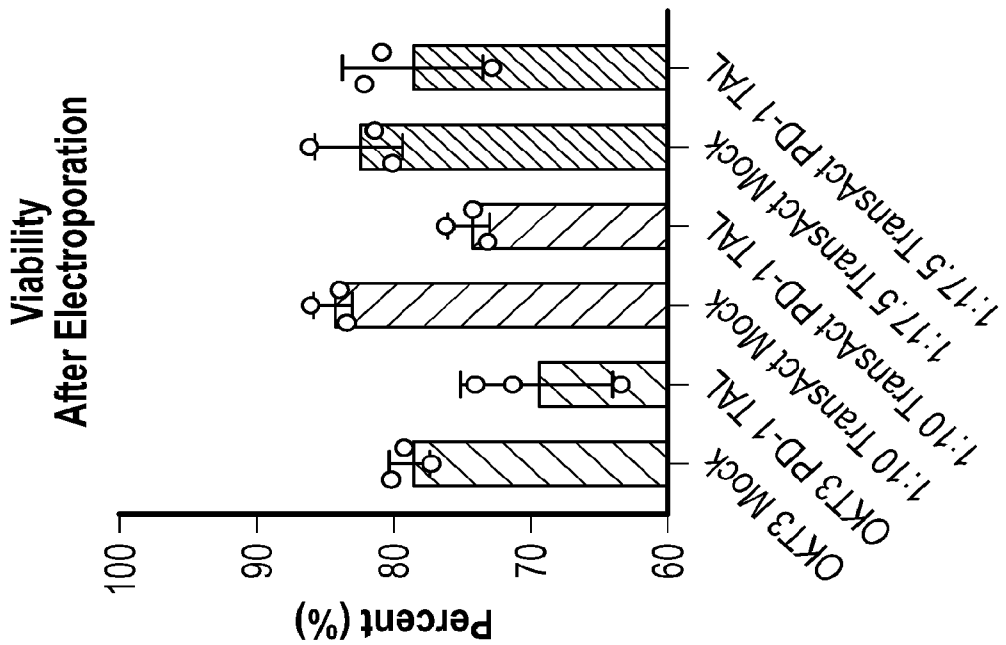

Figure 60C

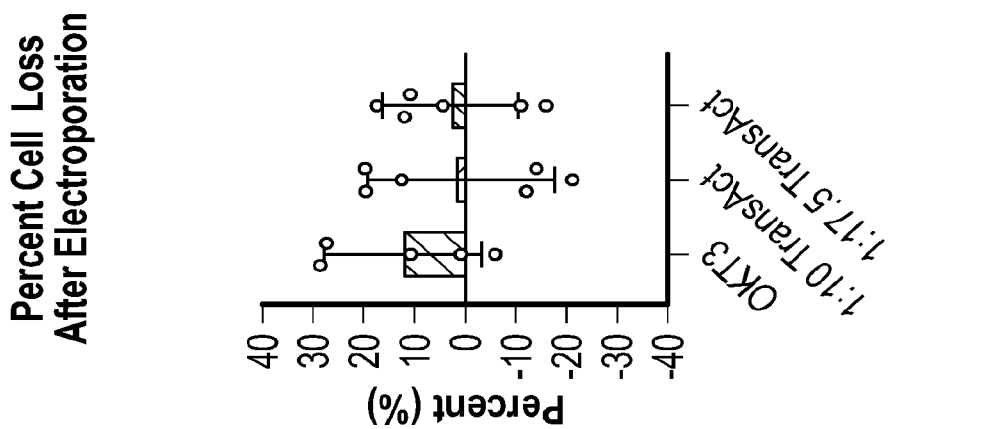

Figure 60B

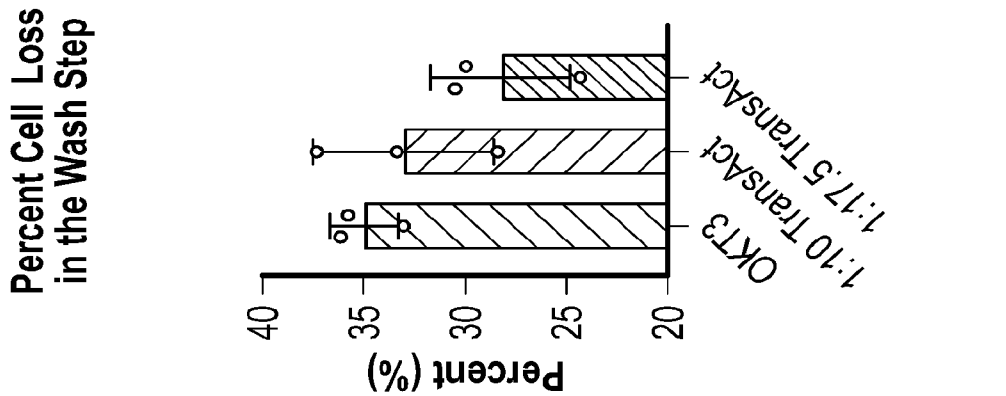

Figure 60A

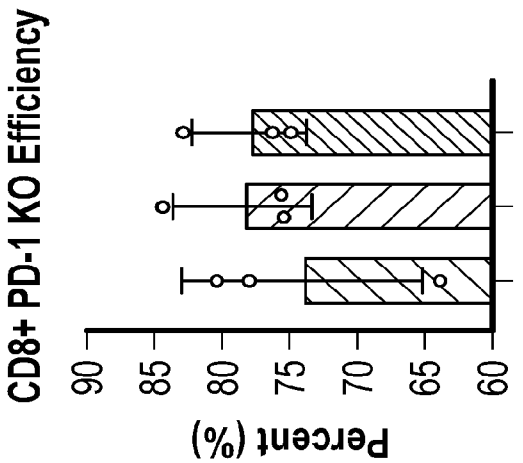

Figure 61C

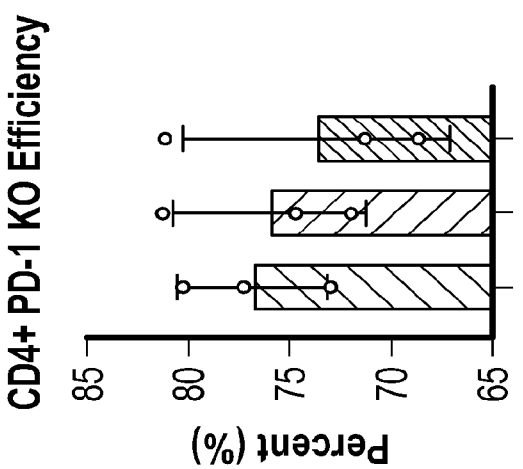

Figure 61B

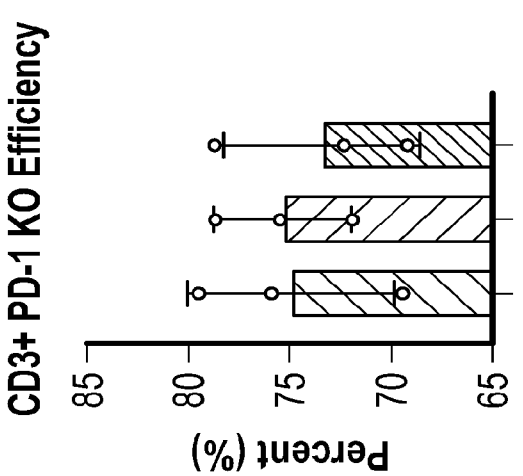

Figure 61A

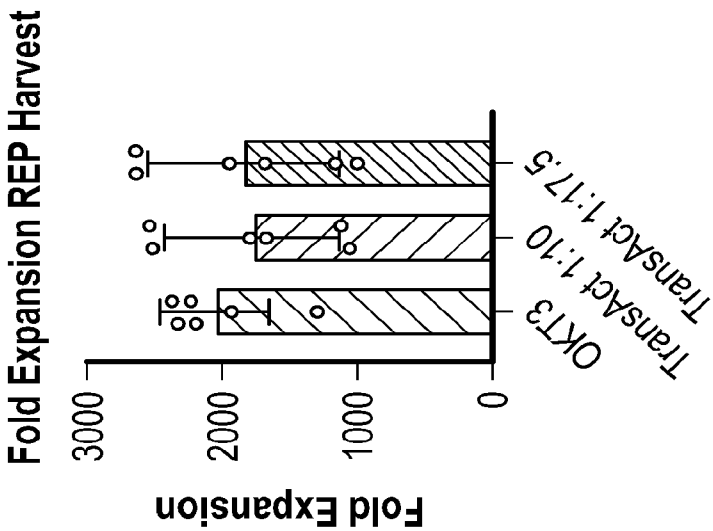

Figure 62B

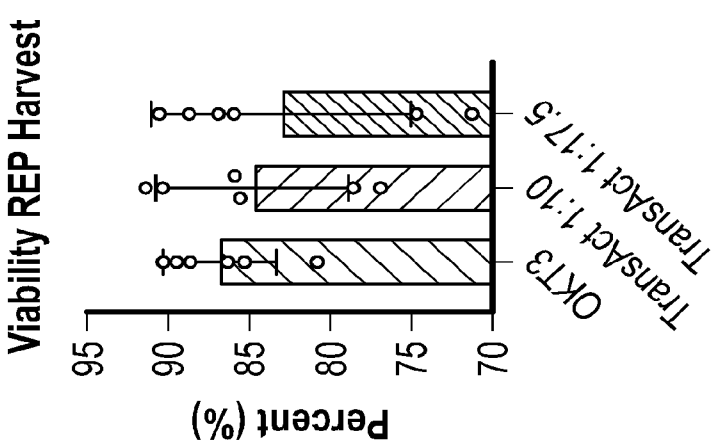

Figure 62A

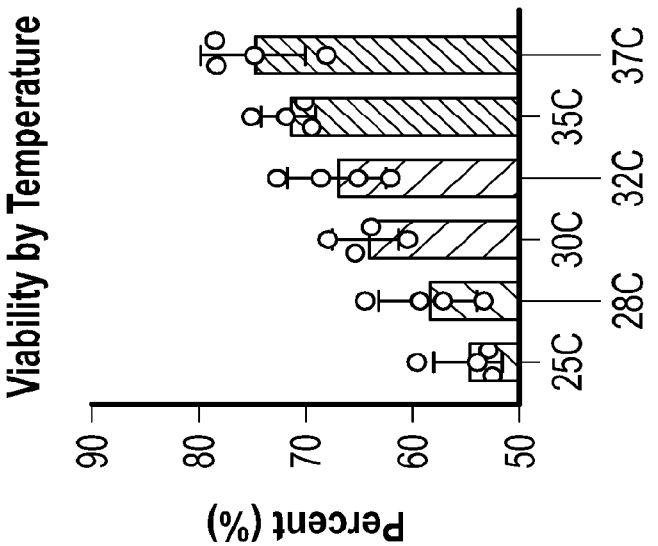

Figure 63B

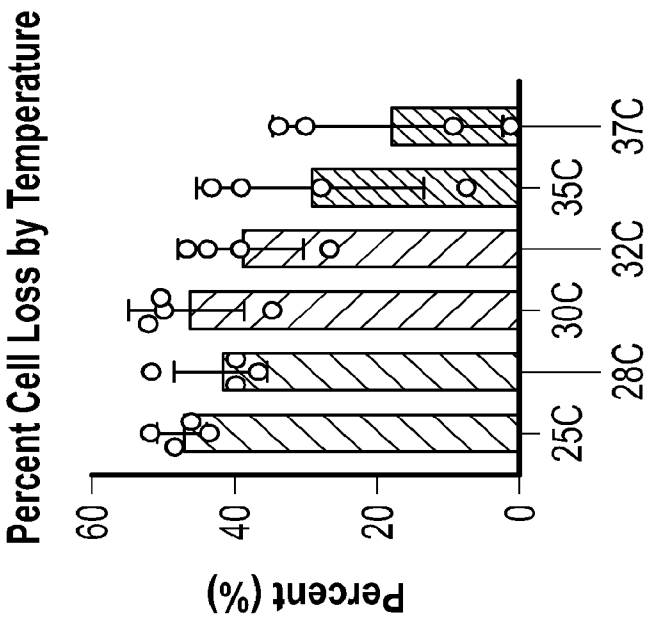

Figure 63A

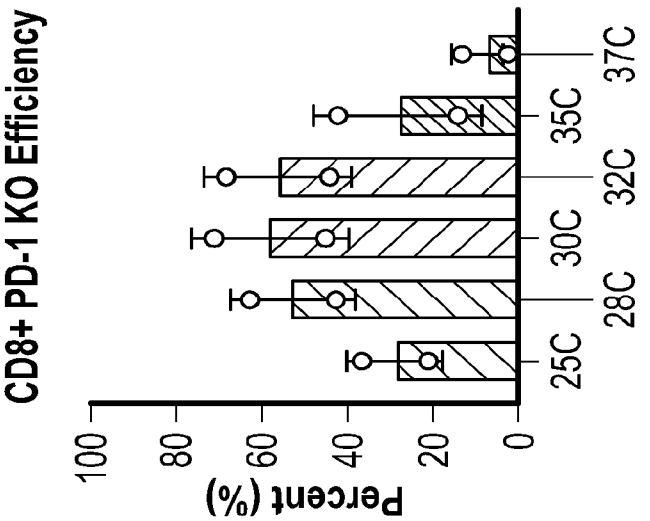

Figure 64C

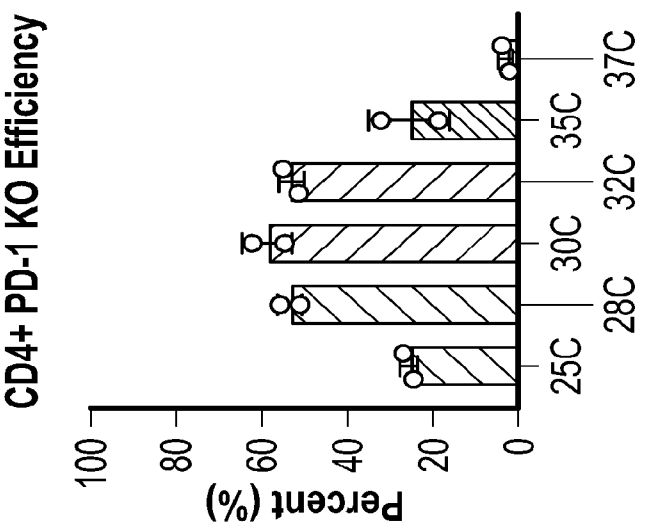

Figure 64B

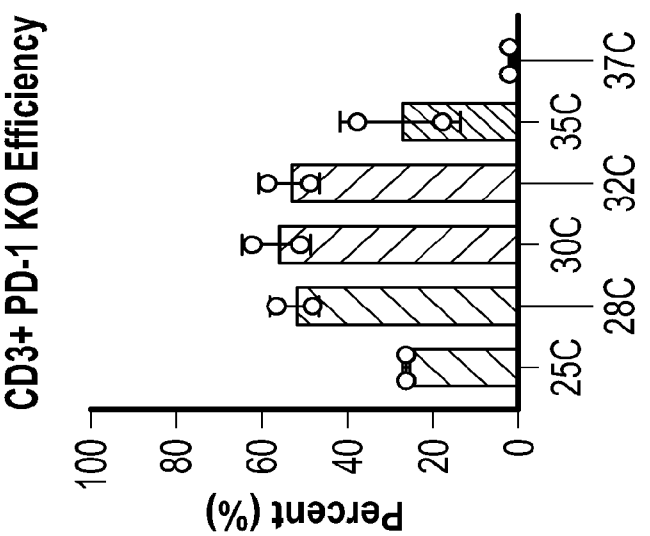

Figure 64A

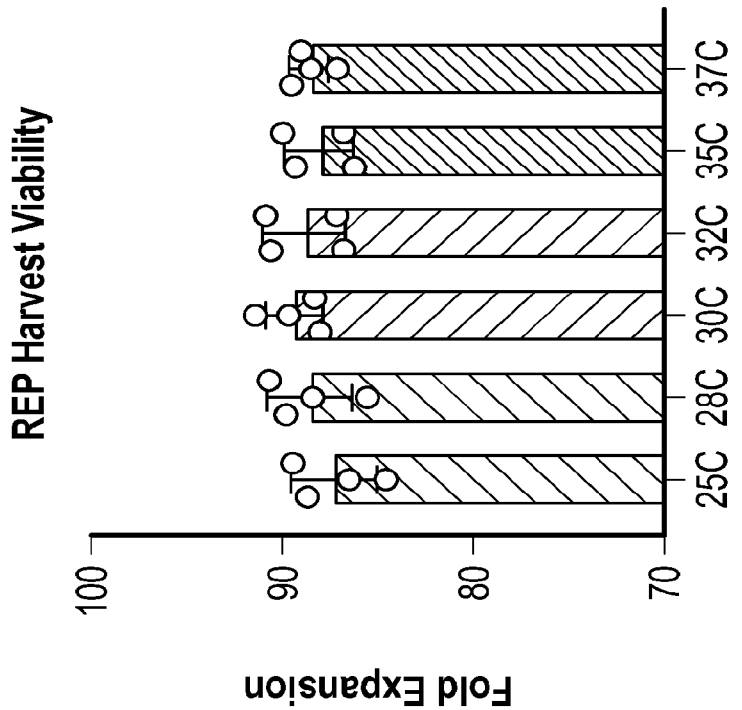

Figure 65B

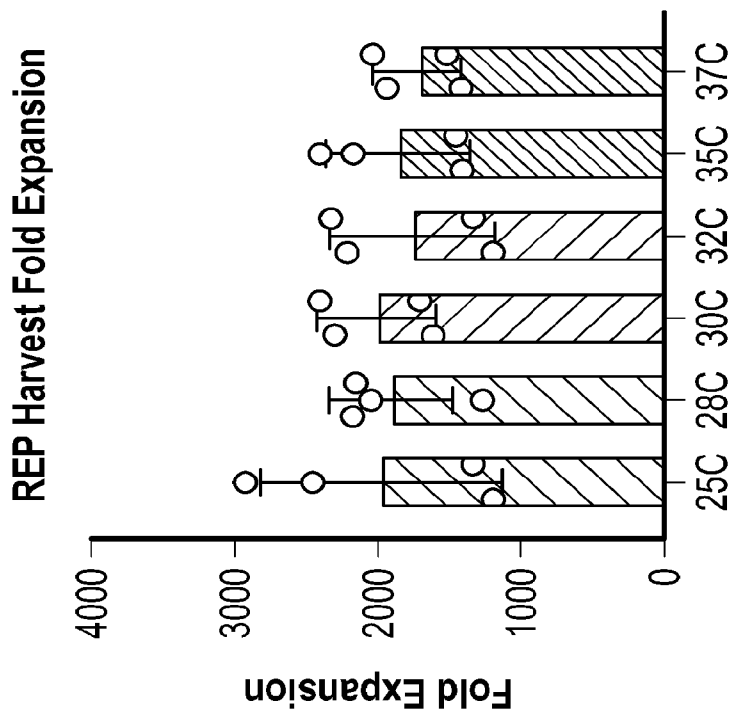

Figure 65A

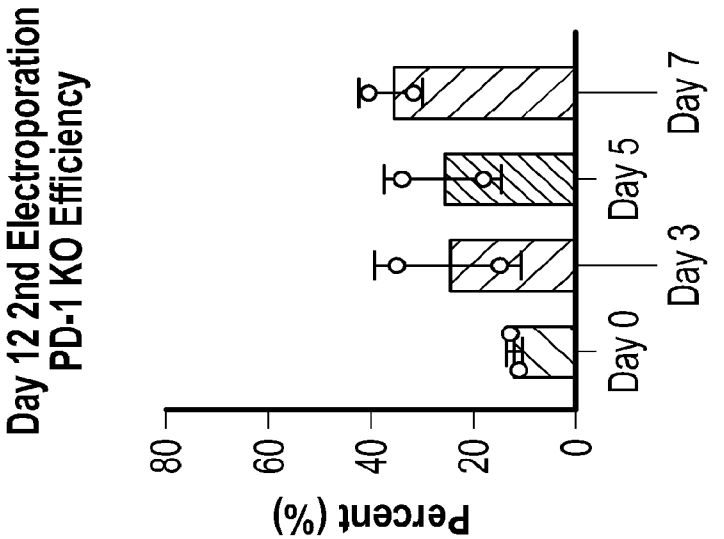

Figure 66C

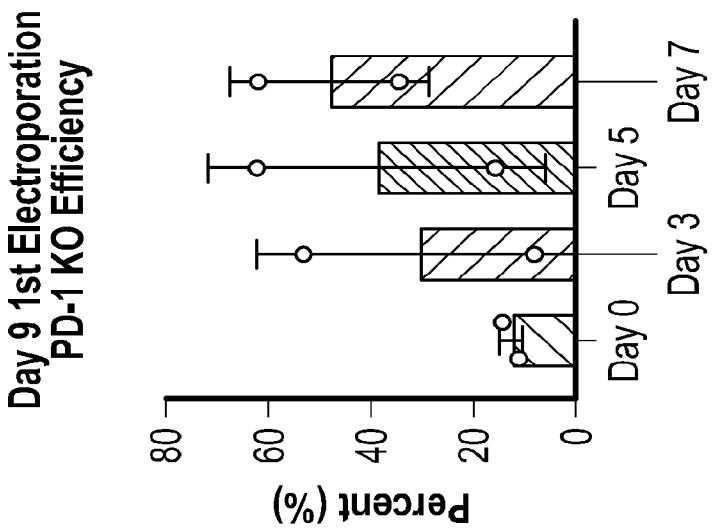

Figure 66B

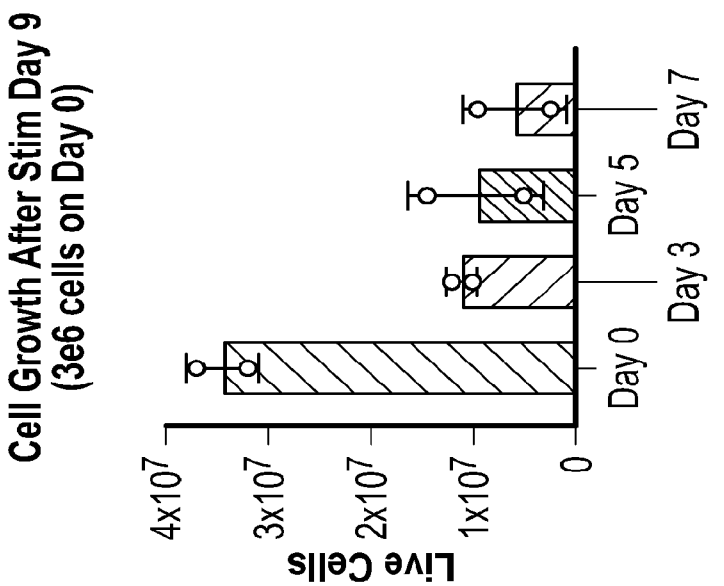

Figure 66A

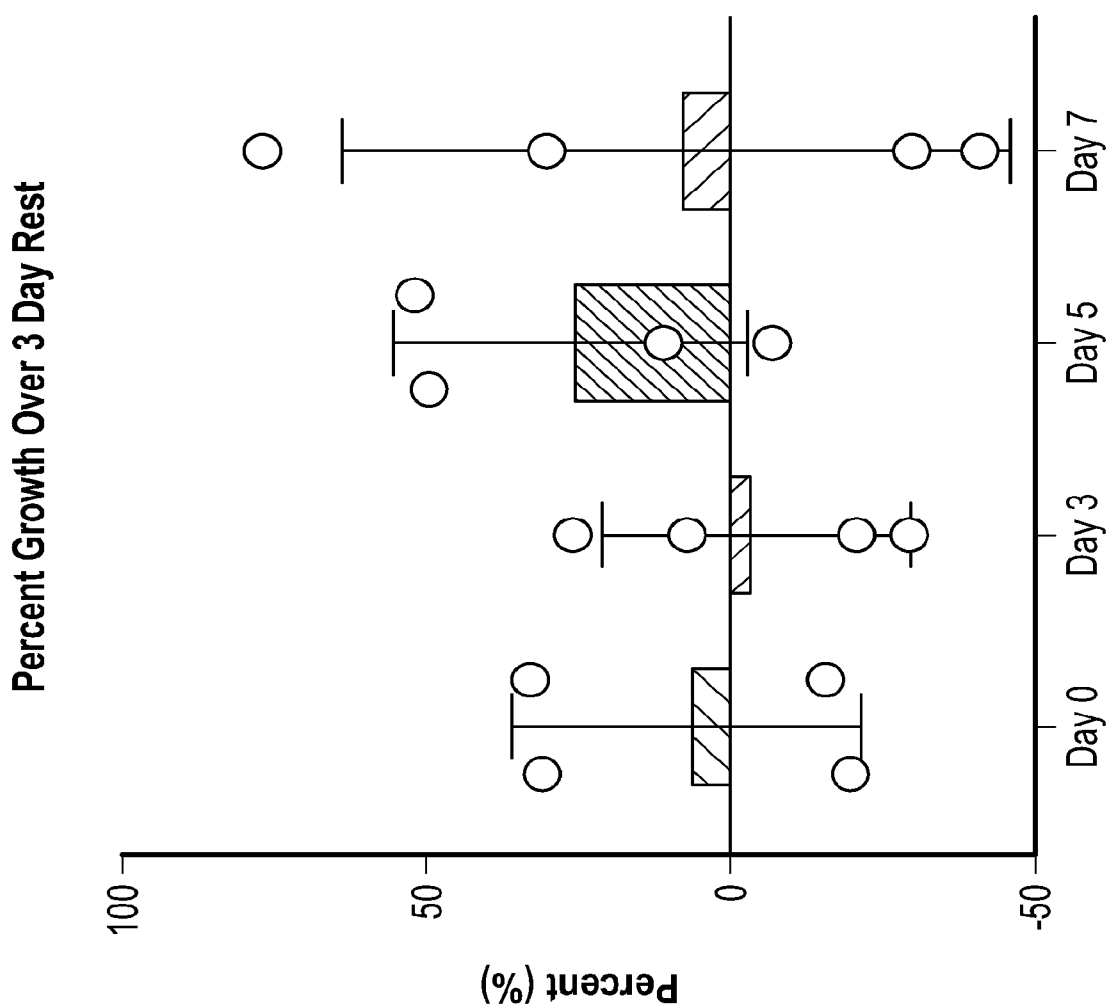
Percent Growth Over 3 Day Rest
Percent (%)
100
50
0
-50
Day 0
Day 3
Day 5
Day 7

Figure 67

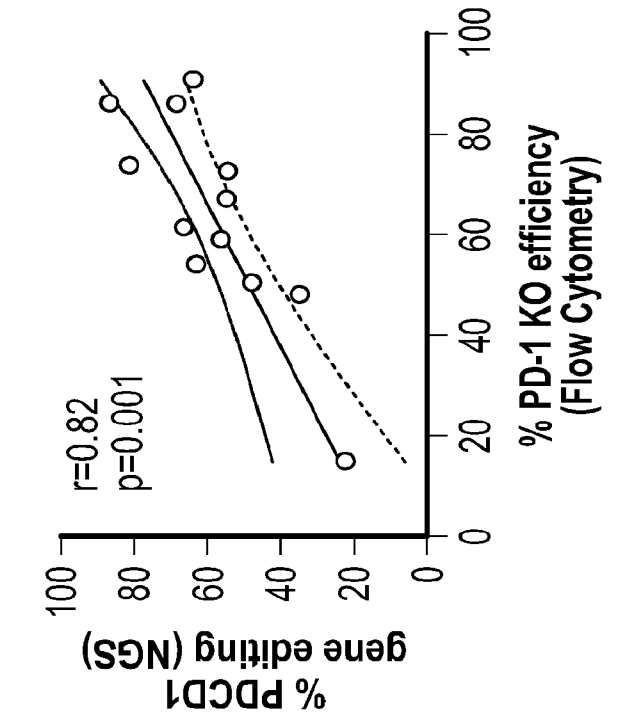

Figure 68C

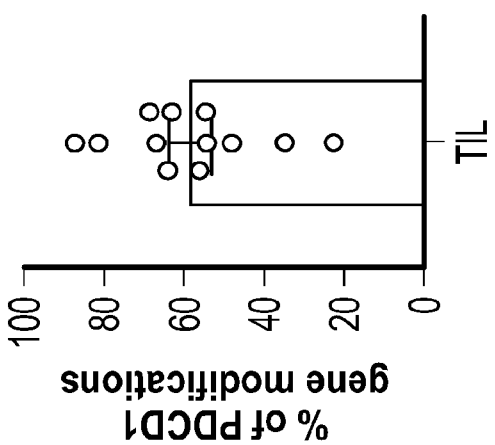

Figure 68B

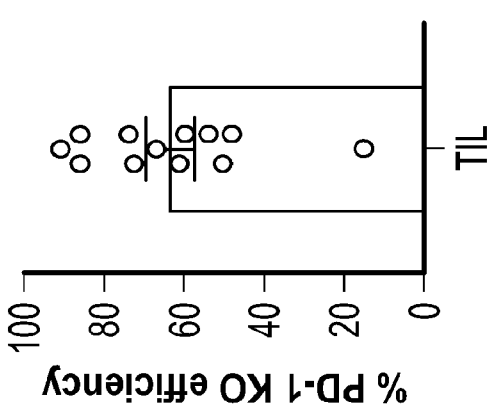

Figure 68A

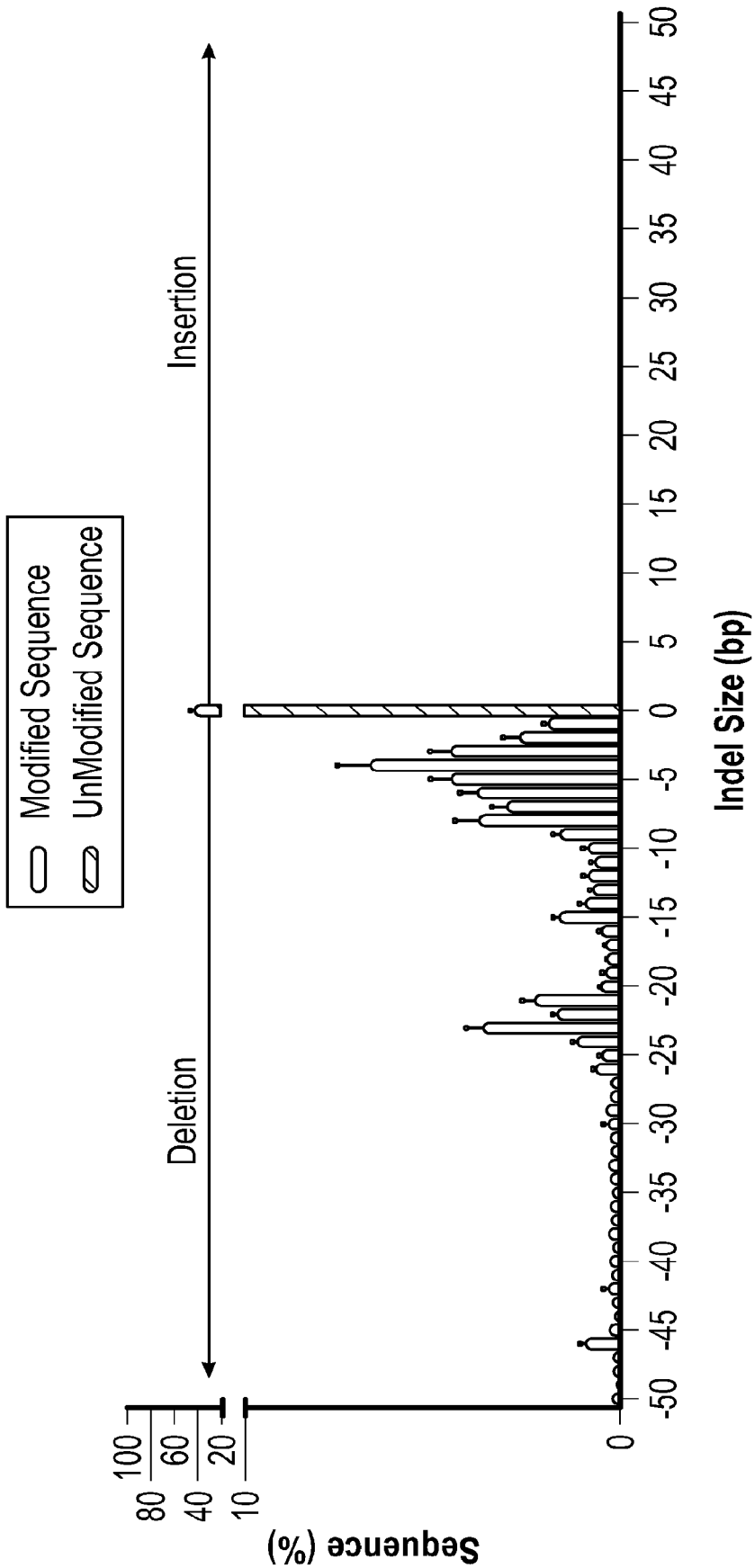
Modified Sequence
UnModified Sequence
Insertion
Deletion
Sequence (%)
100
80
60
40
20
10
0
-50
-45
-40
-35
-30
-25
-20
-15
-10
-5
0
5
10
15
20
25
30
35
40
45
50
Indel Size (bp)

Figure 69

Figure 70A

Figure 70B

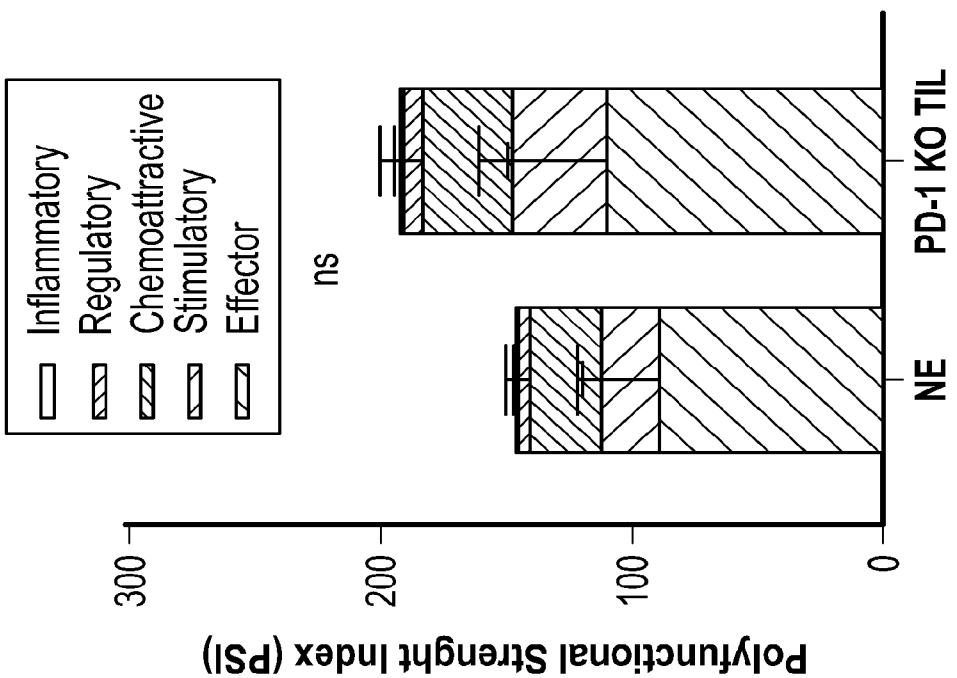

Figure 71B

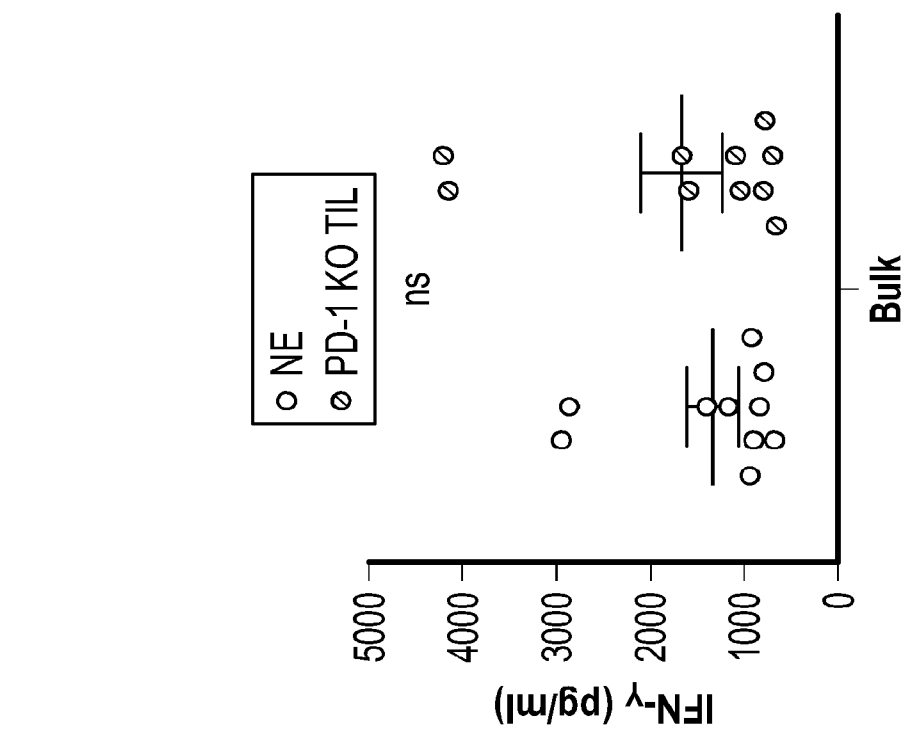

Figure 71A

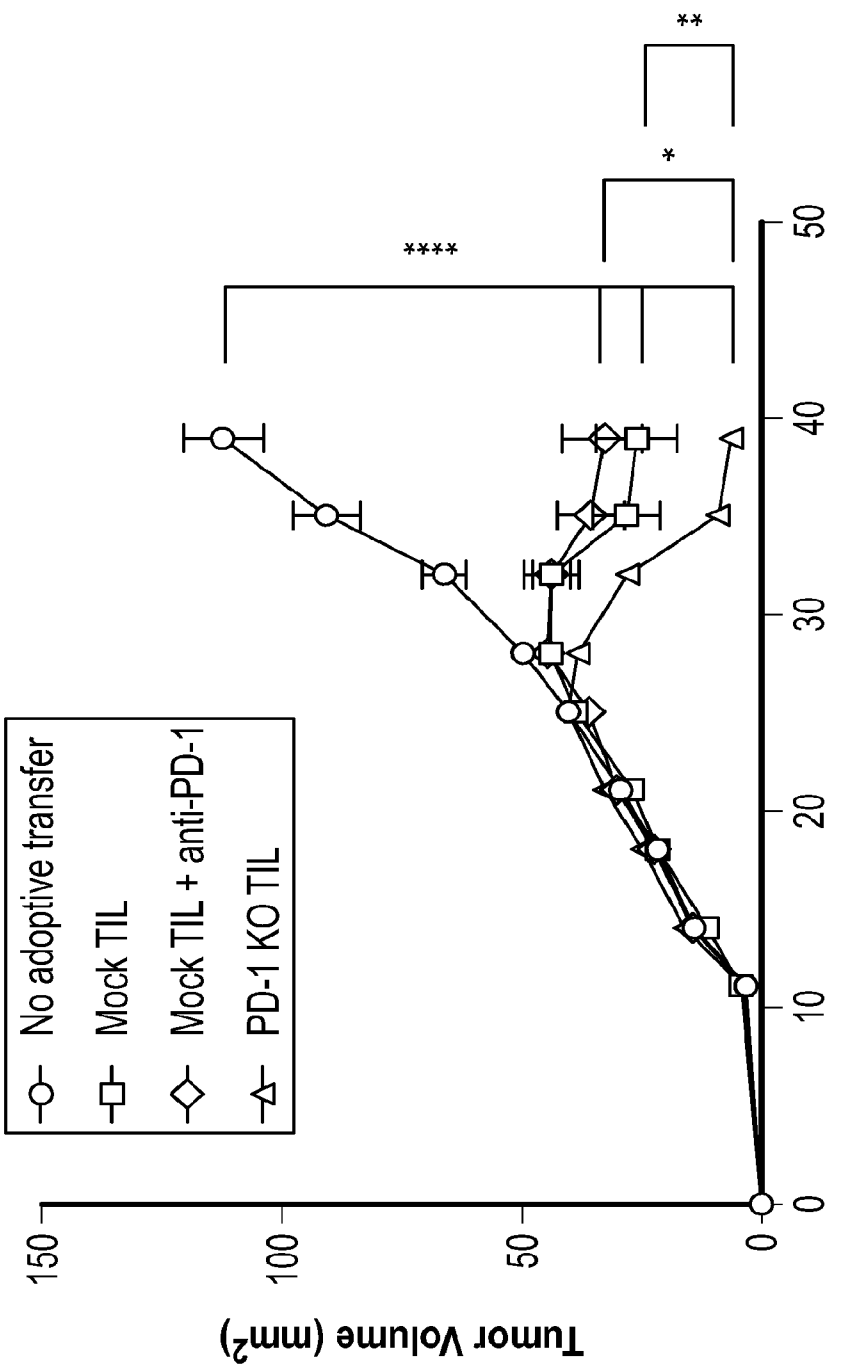
No adoptive transfer
Mock TIL
Mock TIL + anti-PD-1
PD-1 KO TIL
Tumor Volume (mm²)
150
100
50
0
0
10
20
30
40
50
Day
****
*
**

Figure 72

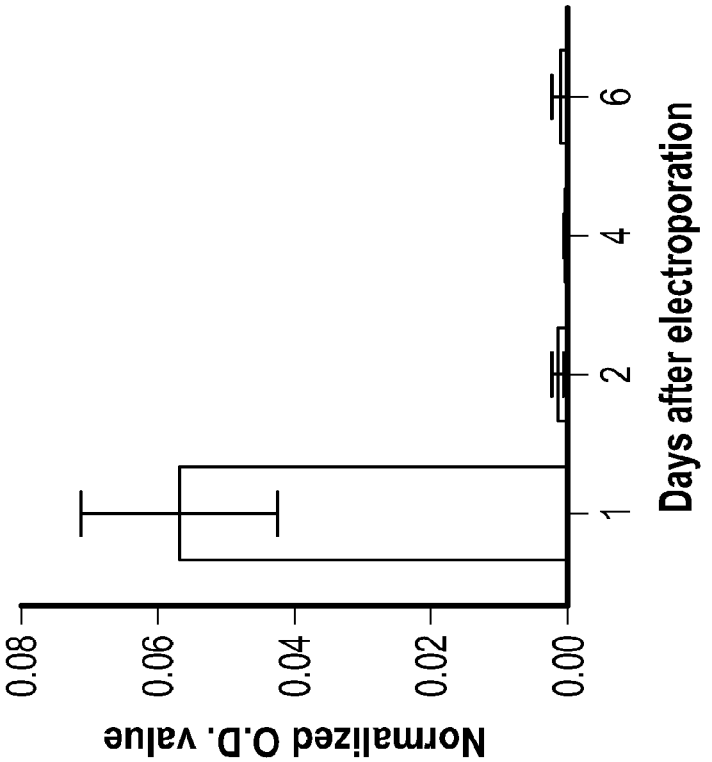

Figure 73B

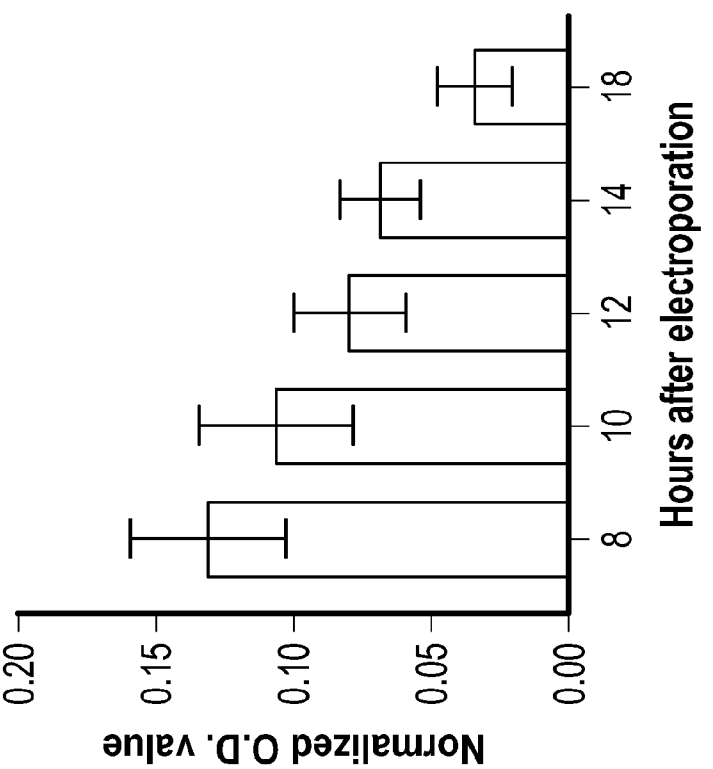

Figure 73A

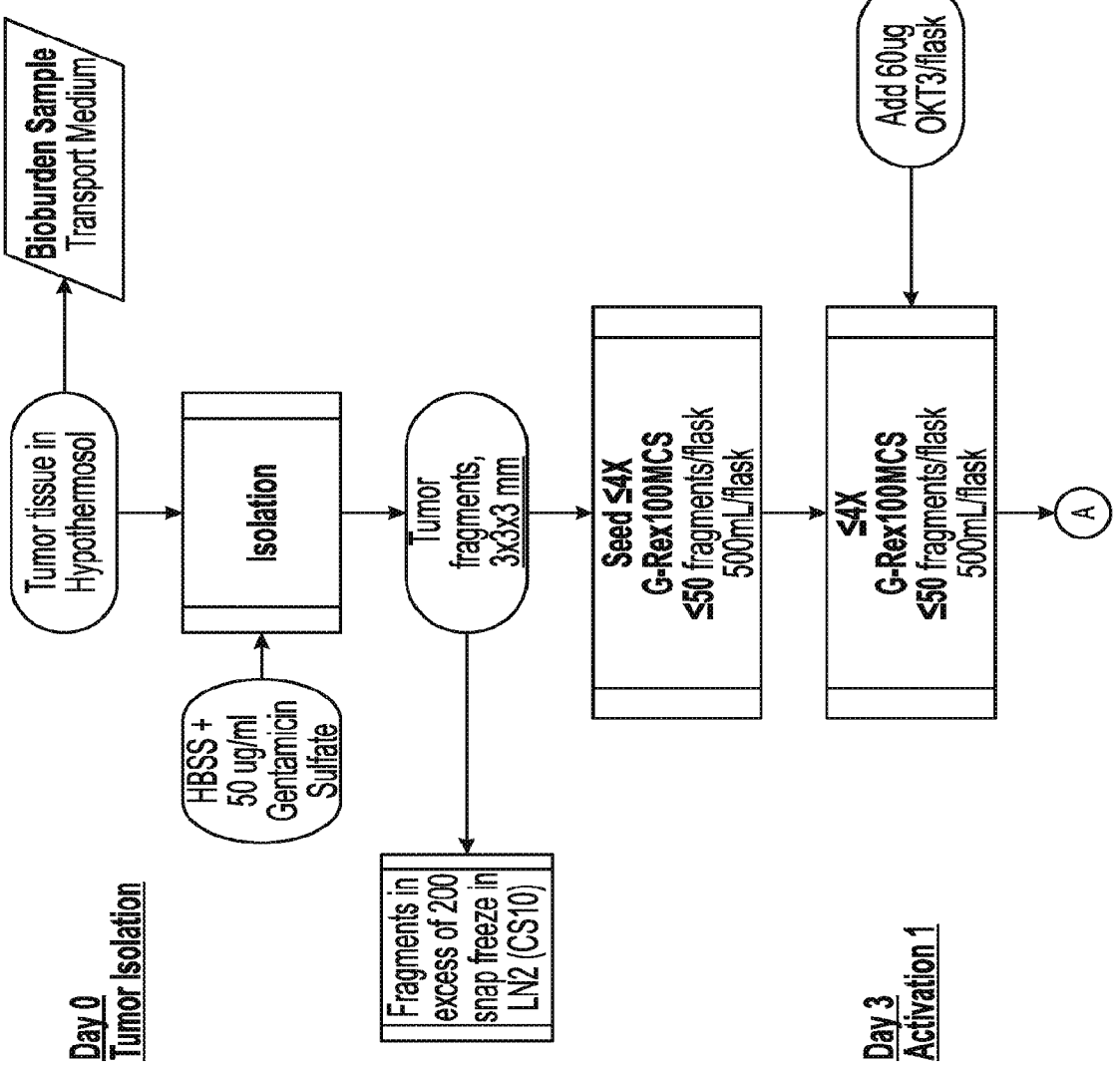
Day 0
Tumor Isolation
Tumor tissue in Hypothermosol
Bioburden Sample
Transport Medium
HBSS + 50 ug/ml Gentamicin Sulfate
Isolation
Tumor fragments, 3x3x3 mm
Fragments in excess of 200 snap freeze in LN2 (CS10)
Seed ≤4X
G-Rex100MCS
≤50 fragments/flask
500mL/flask
Day 3
Activation 1
Add 60ug OKT3/flask
≤4X
G-Rex100MCS
≤50 fragments/flask
500mL/flask
A

Figure 74A

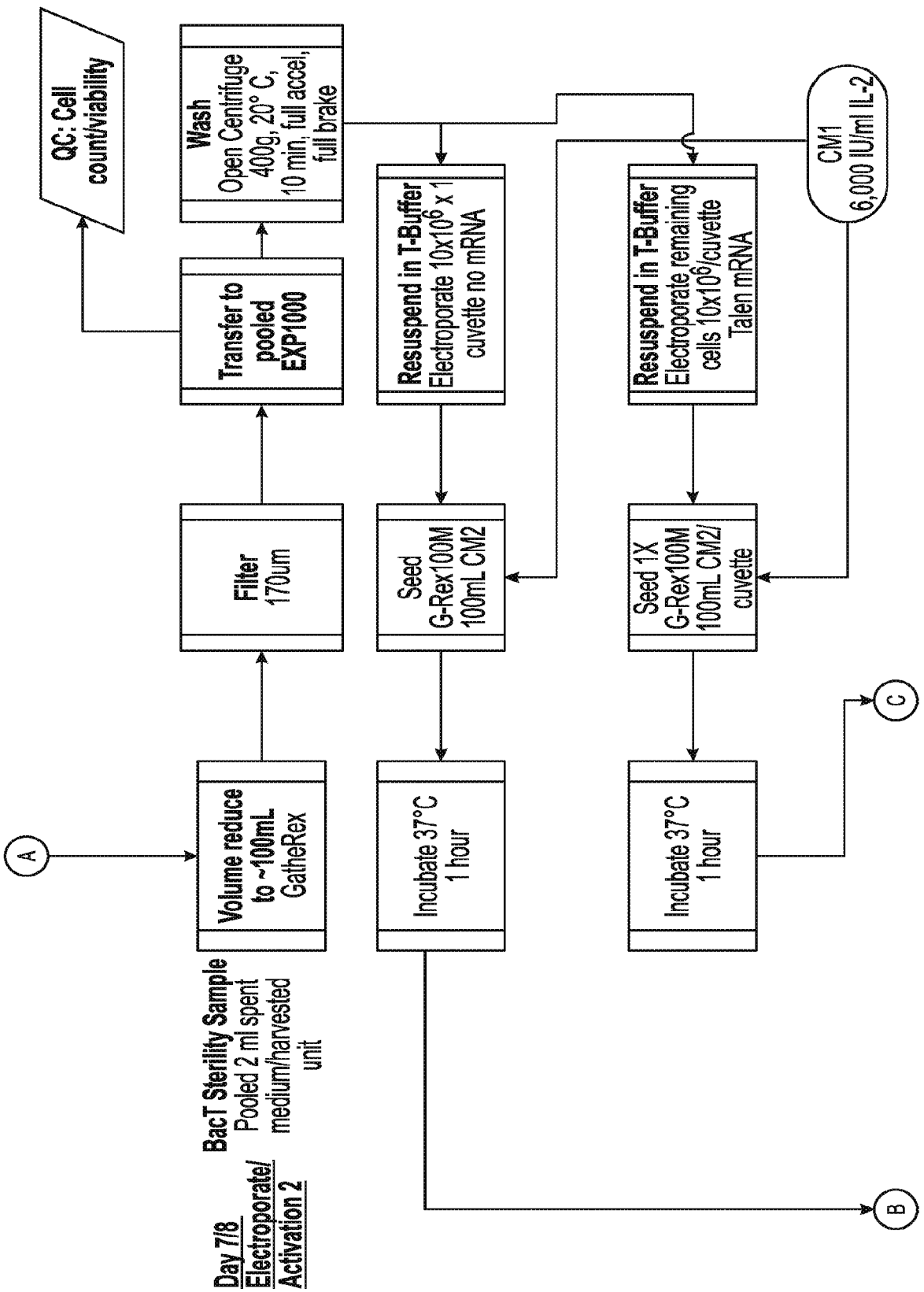
Day 7/8
Electroporate/
Activation 2
BacT Sterility Sample
Pooled 2 ml spent
medium/harvested
unit
A
Volume reduce
to ~100mL
GatheRex
Filter
170um
Transfer to
pooled
EXP1000
QC: Cell
count/viability
Wash
Open Centrifuge
400g, 20° C,
10 min, full accel,
full brake
Seed
G-Rex100M
100mL CM2
Incubate 37°C
1 hour
Resuspend in T-Buffer
Electroporate 10x10^6 x 1
cuvette no mRNA
Seed 1X
G-Rex100M
100mL CM2/
cuvette
Incubate 37°C
1 hour
Resuspend in T-Buffer
Electroporate remaining
cells 10x10^6/cuvette
Talen mRNA
CM1
6,000 IU/ml IL-2
B
C

Figure 74B

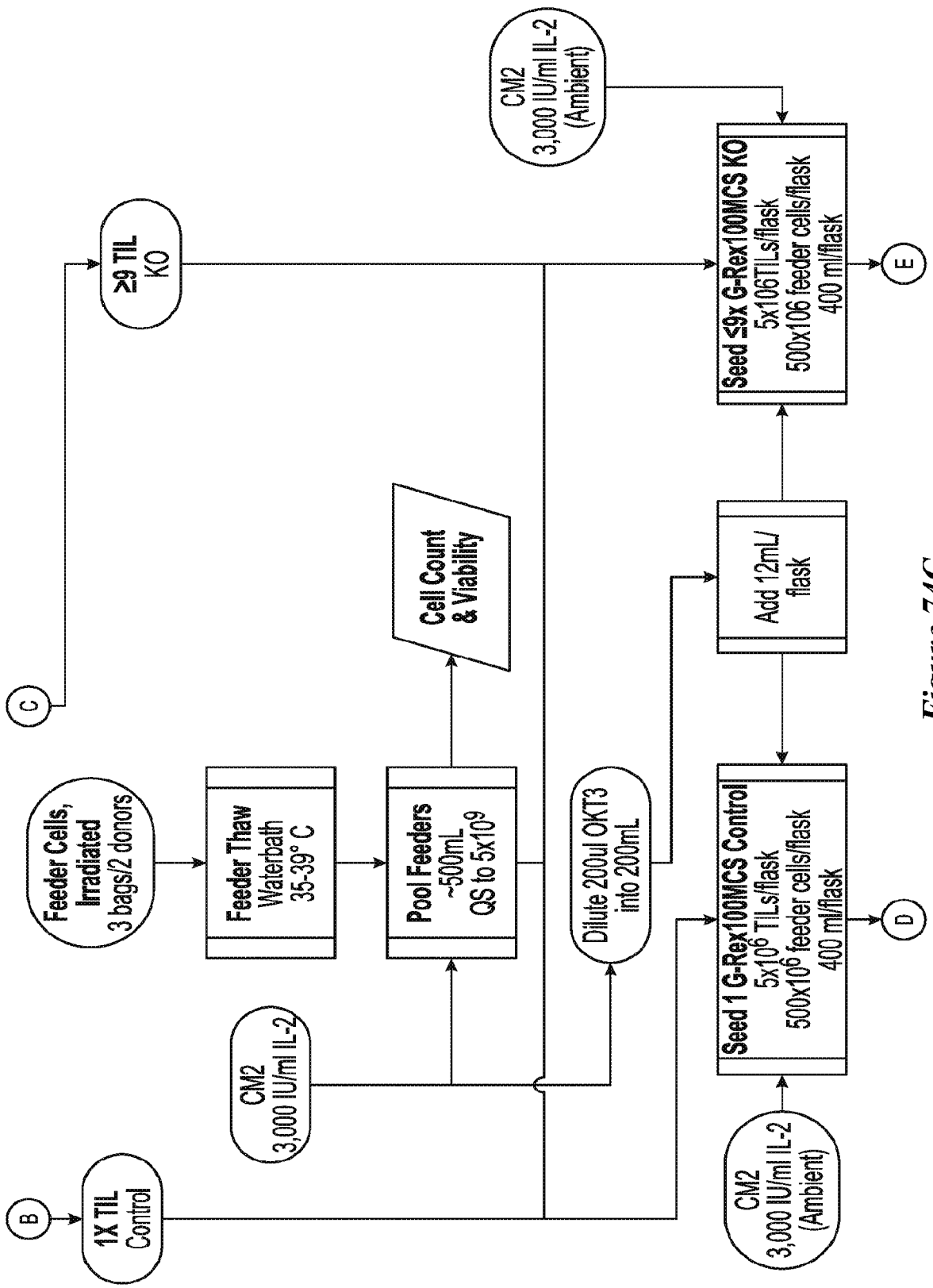
B
1X TIL Control
C
≥9 TIL KO
Feeder Cells, Irradiated 3 bags/2 donors
Feeder Thaw Waterbath 35-39° C
Pool Feeders ~500mL QS to 5x10^9
Cell Count & Viability
CM2 3,000 IU/ml IL-2
CM2 3,000 IU/ml IL-2 (Ambient)
Dilute 200ul OKT3 into 200mL
Add 12mL/ flask
Seed 1 G-Rex100MCS Control 5x10^6 TILs/flask 500x10^6 feeder cells/flask 400 ml/flask
Seed ≤9x G-Rex100MCS KO 5x10^6TILs/flask 500x10^6 feeder cells/flask 400 ml/flask
CM2 3,000 IU/ml IL-2 (Ambient)
D
E

Figure 74C

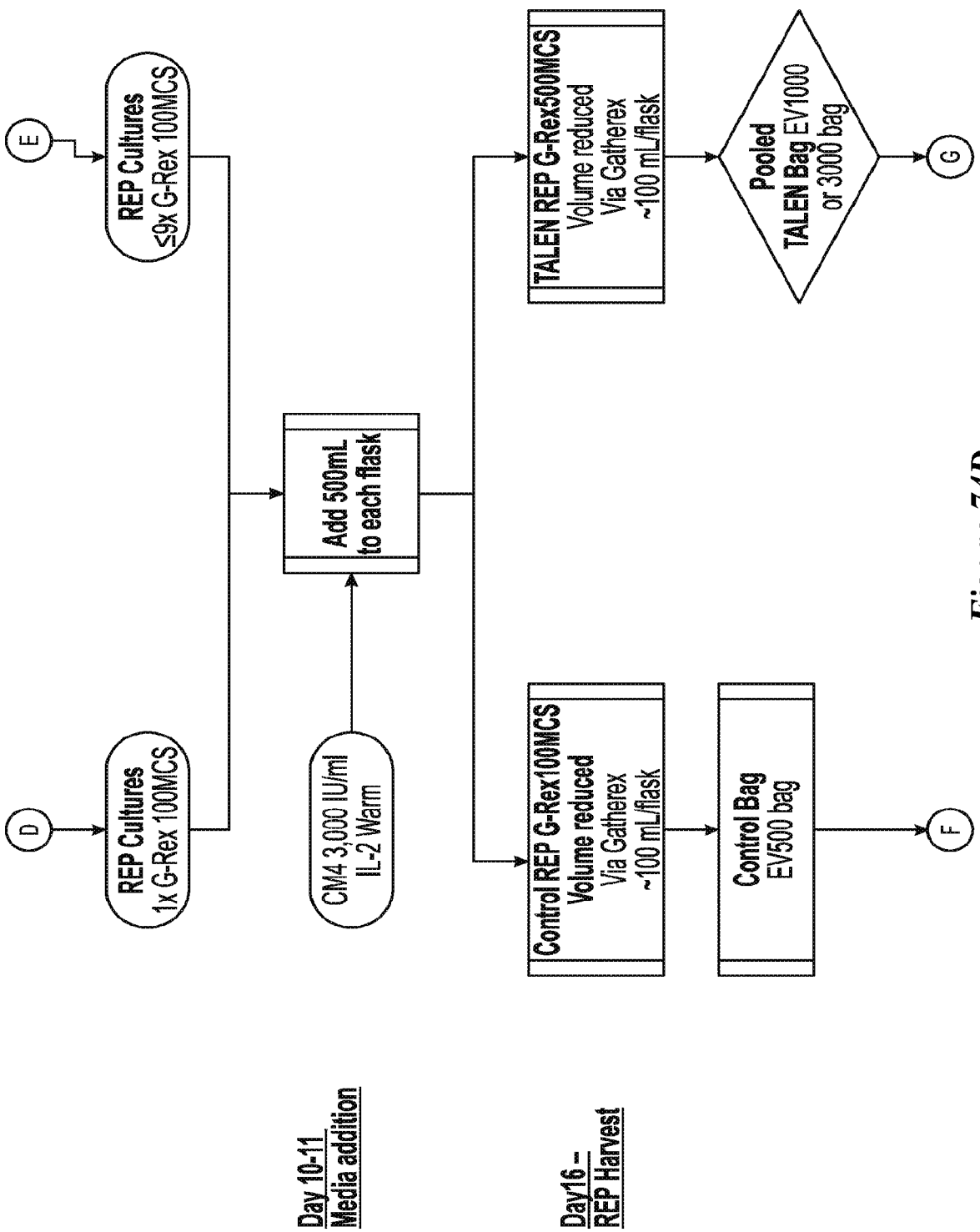
D
REP Cultures
1x G-Rex 100MCS
E
REP Cultures
≤9x G-Rex 100MCS
CM4 3,000 IU/ml
IL-2 Warm
Add 500mL
to each flask
Day 10-11
Media addition
Control REP G-Rex100MCS
Volume reduced
Via Gatherex
~100 mL/flask
TALEN REP G-Rex500MCS
Volume reduced
Via Gatherex
~100 mL/flask
Control Bag
EV500 bag
Pooled
TALEN Bag EV1000
or 3000 bag
F
G
Day16 –
REP Harvest

Figure 74D

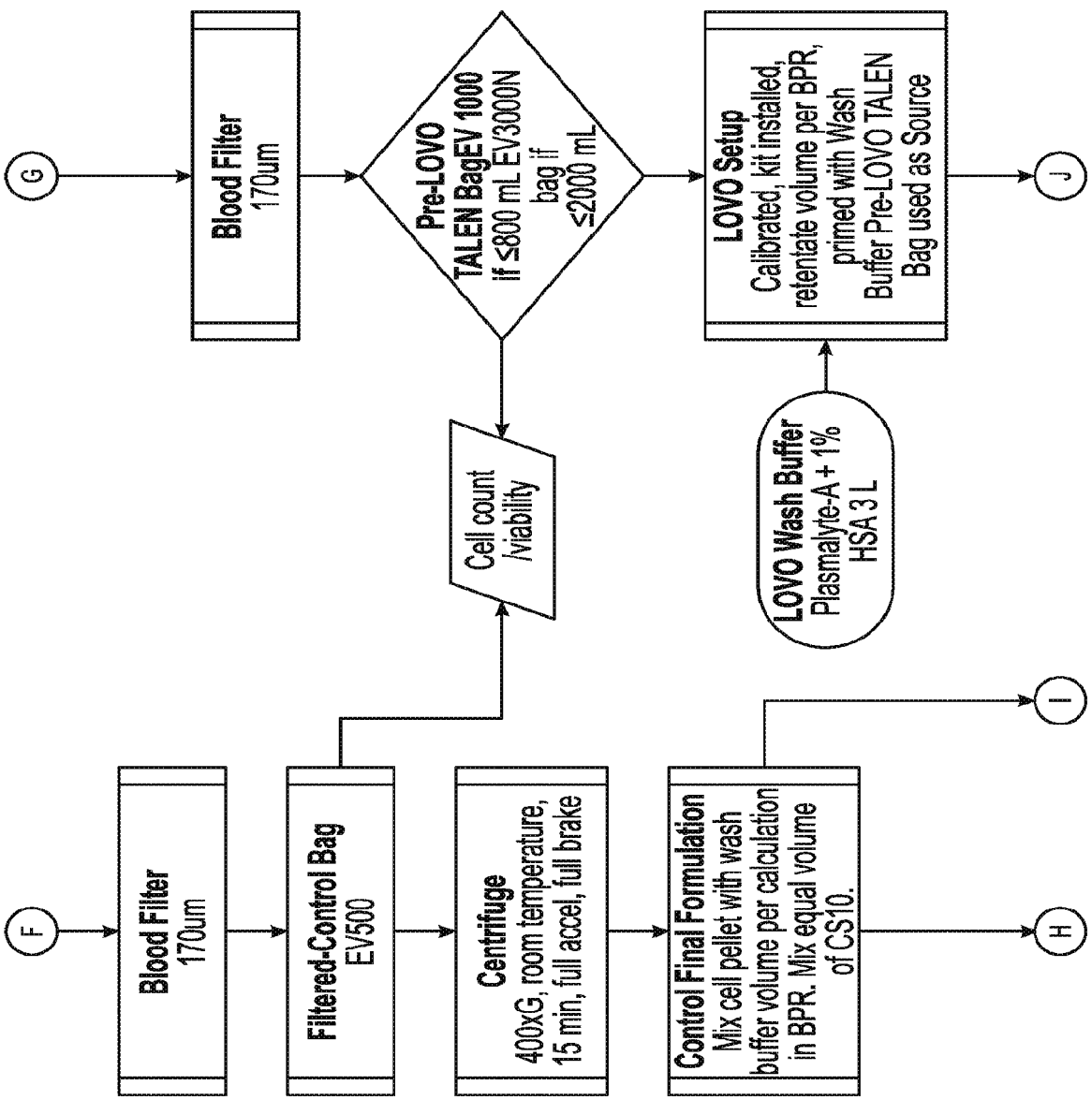
F
Blood Filter
170um
Filtered-Control Bag
EV500
Centrifuge
400xG, room temperature,
15 min, full accel, full brake
Control Final Formulation
Mix cell pellet with wash
buffer volume per calculation
in BPR. Mix equal volume
of CS10.
H
I
Cell count
/viability
LOVO Wash Buffer
Plasmalyte-A + 1%
HSA 3 L
G
Blood Filter
170um
Pre-LOVO
TALEN BagEV 1000
if ≤800 mL EV3000N
bag if
≤2000 mL
LOVO Setup
Calibrated, kit installed,
retentate volume per BPR,
primed with Wash
Buffer Pre-LOVO TALEN
Bag used as Source
J

Figure 74E

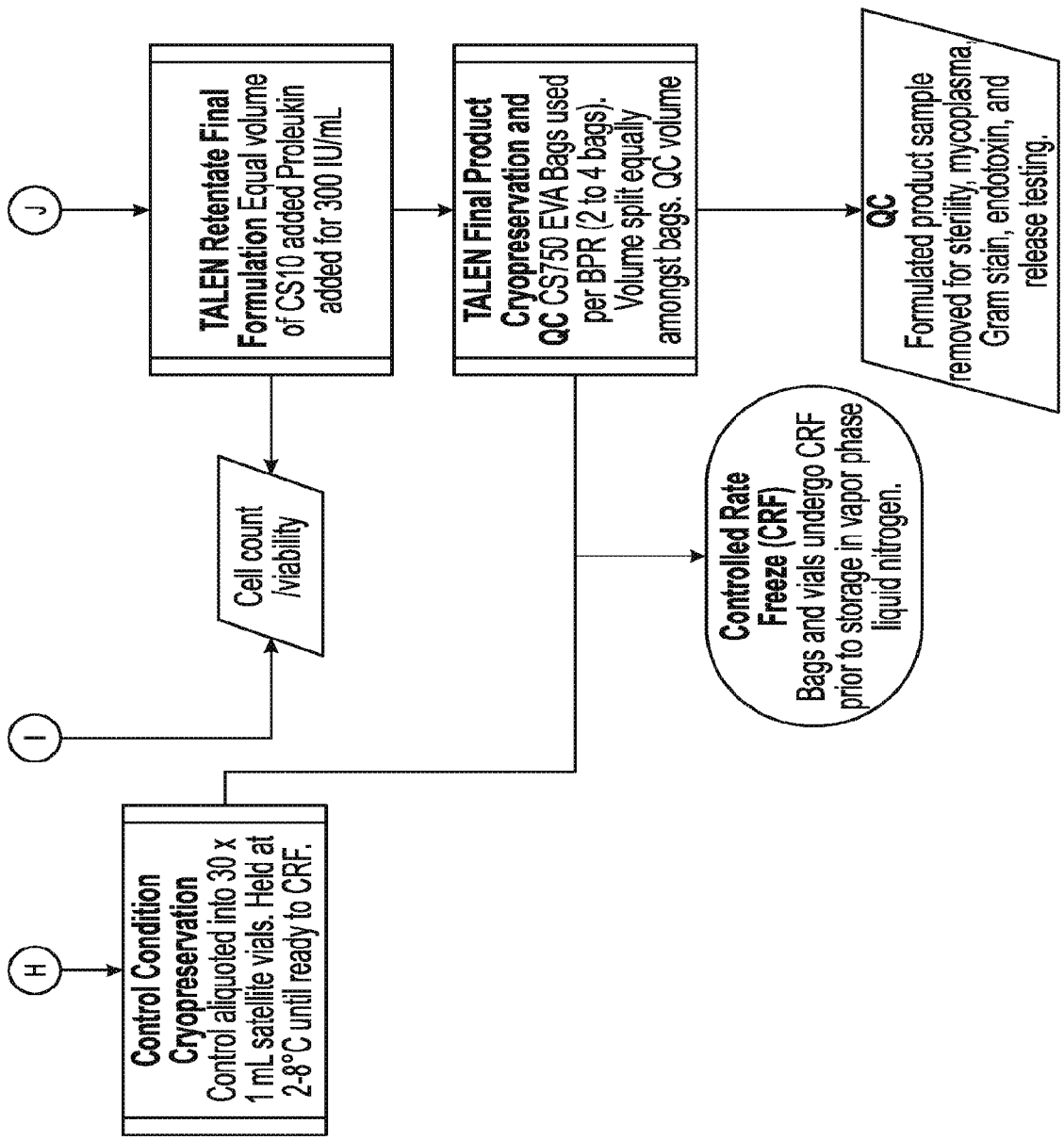
J
TALEN Retentate Final Formulation Equal volume of CS10 added Proleukin added for 300 IU/mL
TALEN Final Product Cryopreservation and QC CS750 EVA Bags used per BPR (2 to 4 bags). Volume split equally amongst bags. QC volume
QC
Formulated product sample removed for sterility, mycoplasma, Gram stain, endotoxin, and release testing.
I
Cell count /viability
Controlled Rate Freeze (CRF)
Bags and vials undergo CRF prior to storage in vapor phase liquid nitrogen.
H
Control Condition Cryopreservation
Control aliquoted into 30 x 1 mL satellite vials. Held at 2-8°C until ready to CRF.

Figure 74F

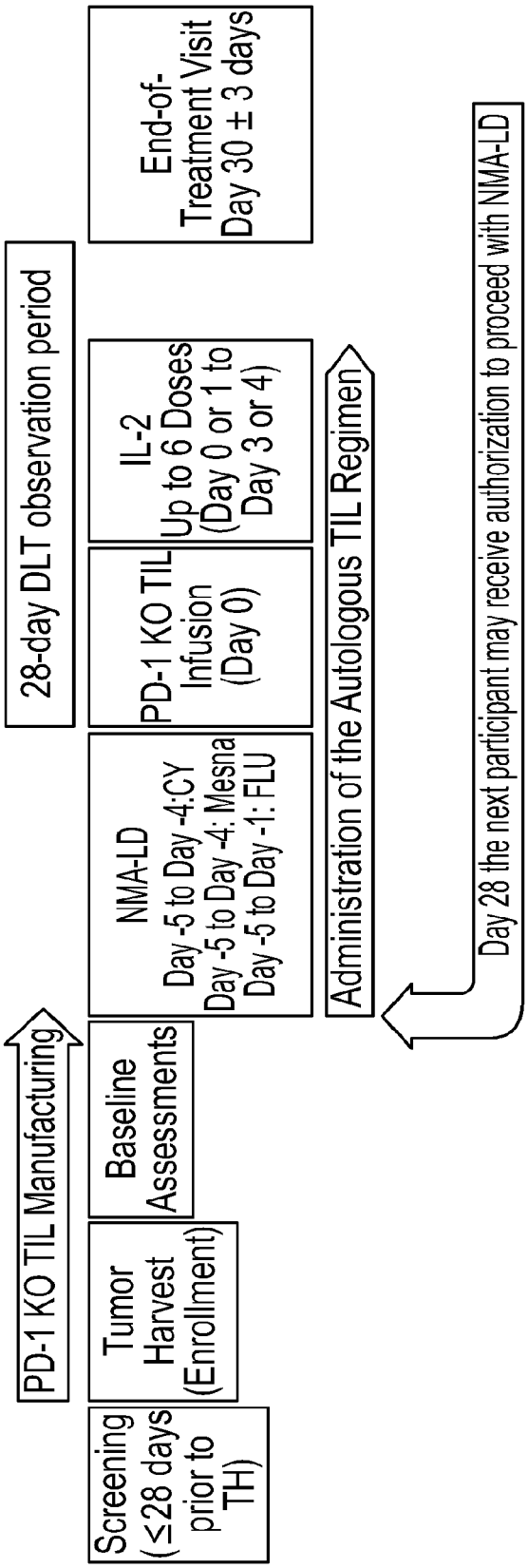
Screening (≤28 days prior to TH)
Tumor Harvest (Enrollment)
Baseline Assessments
PD-1 KO TIL Manufacturing
NMA-LD Day -5 to Day -4:CY Day -5 to Day -4: Mesna Day -5 to Day -1: FLU
PD-1 KO TIL Infusion (Day 0)
IL-2 Up to 6 Doses (Day 0 or 1 to Day 3 or 4)
28-day DLT observation period
End-of-Treatment Visit Day 30 ± 3 days
Administration of the Autologous TIL Regimen
Day 28 the next participant may receive authorization to proceed with NMA-LD

Figure 75A

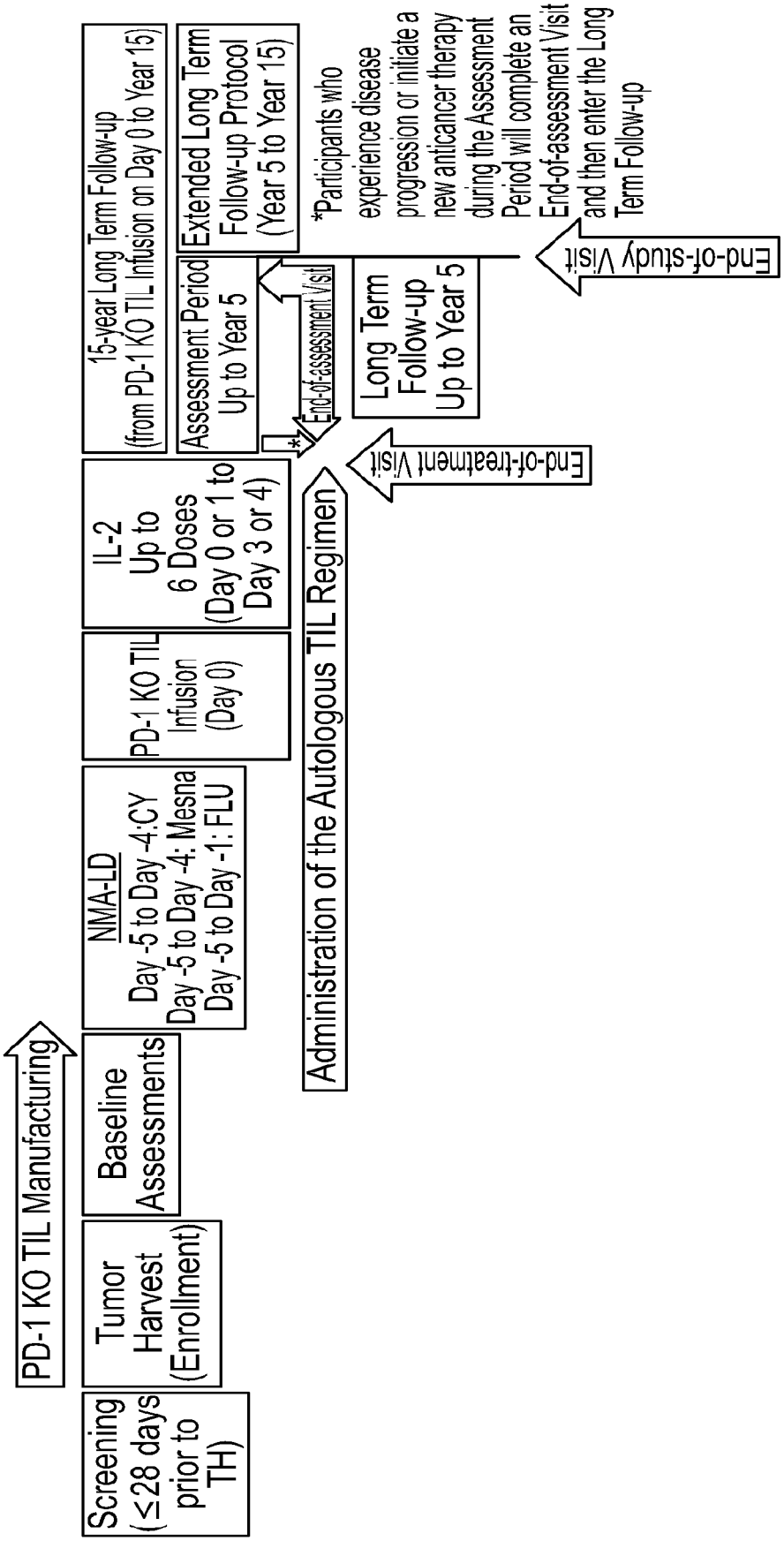
Screening (≤28 days prior to TH)
Tumor Harvest (Enrollment)
Baseline Assessments
PD-1 KO TIL Manufacturing
NMA-LD
Day -5 to Day -4:CY
Day -5 to Day -4: Mesna
Day -5 to Day -1: FLU
PD-1 KO TIL Infusion (Day 0)
IL-2 Up to 6 Doses (Day 0 or 1 to Day 3 or 4)
Administration of the Autologous TIL Regimen
End-of-treatment Visit
Assessment Period Up to Year 5
End-of-assessment Visit
Long Term Follow-up Up to Year 5
15-year Long Term Follow-up (from PD-1 KO TIL Infusion on Day 0 to Year 15)
Extended Long Term Follow-up Protocol (Year 5 to Year 15)
End-of-study Visit
*Participants who experience disease progression or initiate a new anticancer therapy during the Assessment Period will complete an End-of-assessment Visit and then enter the Long Term Follow-up

Figure 75B

EXPERIMENT	ELECTROPORATION SETTINGS TESTED	CELL CONCENTRATIONS TESTED	RECOVERY	GFP EXPRESSION	PD-1 KO EFFICIENCY	REP HARVEST TVC	COMPARISON TO BTX
DEMO DAY	THERMO OPTIMIZATION PROGRAMS	20E6/ML	HIGHEST WITH 1700/20/1	HIGHEST WITH 1700/20/1 AND 2300/3/4	N/A	N/A	* NOT TESTED
NEON EXP 1	PULSE DURATION AND NUMBER VARIATIONS ON 1700/20/1 AND 2300/3/4	20E6/ML	HIGHEST WITH 1700/10/1 AND 2300/2/3	HIGHEST WITH 2300/3/4	N/A	N/A	* NOT TESTED
XENON EXP 1	2300/3/4	1E6/ML 5E6/ML 10E6/ML 2E6/ML	DECREASED WITH LOWER CELL CONCENTRATION	SIMILAR ACROSS CELL CONCENTRATIONS	N/A	N/A	LOWER GFP AND RECOVERY THAN BTX
XENON EXP 3	1400/30/1 1700/20/1 2300/2/3 2500/2/5	5E6/ML 25E6/ML	HIGHEST WITH 2300/2/3 (SAME BETWEEN 5E6 AND 25E6)	HIGHEST WITH 2300/2/3 AND 2500/2/5 (SAME BETWEEN 5E6 AND 25E6)	N/A	N/A	SIMILAR GFP AND RECOVERY TO BTX
XENON EXP 4	2300/2/3	25E6/ML	HIGHER WITH XENON THAN BTX	N/A	89% XENON 85% BTX	1.04E10 XENON TALEN 1.21E10 BTX TALEN	HIGHER PD-1 KO EFFICIENCY AND RECOVERY THAN BTX, SIMILAR CELL DOUBLING

Figure 76

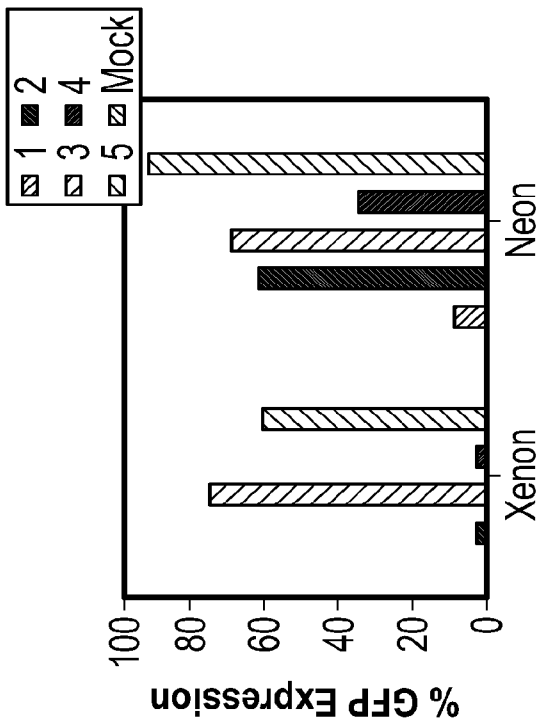

Figure 77A

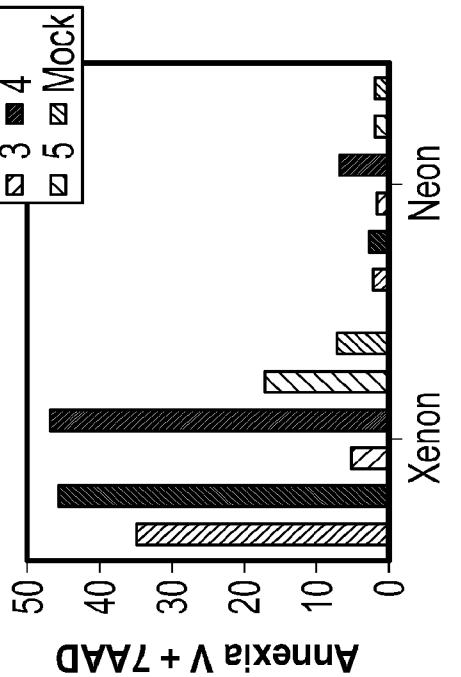

Figure 77B

Electroporation Settings					
Pulse Profile ID	Voltage (V)	Duration (ms)	Pulses	Neon Pulse Interval (ms)	Xenon Pulse Interval (ms)
1	1150	30	2	1000	500
2	1600	10	3	1000	500
3	1700	20	1	1000	500
4	1400	20	2	1000	500
5	2300	3	4	1000	500
6	1400	30	1	1000	500

Figure 77C

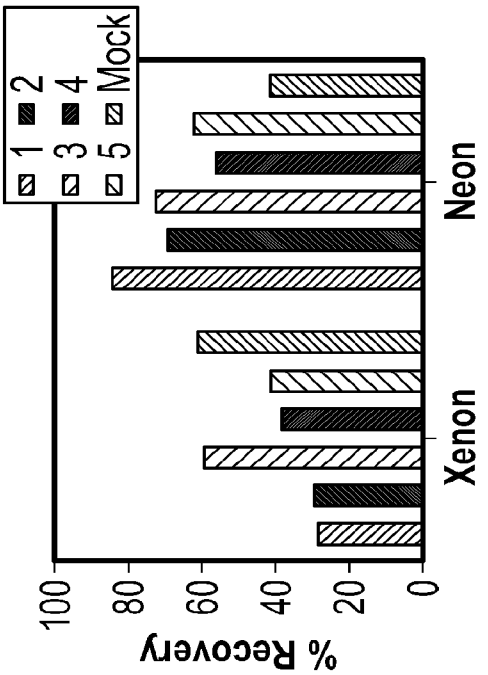

Figure 77D

Electroporation Settings				
Pulse Profile ID	Voltage (V)	Duration (ms)	Pulses	Neon Pulse Interval (ms)
1	1700	20	1	1000
2	1700	30	1	1000
3	1700	10	1	1000
4	1700	5	1	1000
5*	2300	3	4	1000
6	2300	2	4	1000
7	2300	5	4	1000
8	2300	3	3	1000
9	2300	2	3	1000
10	2300	5	3	1000

Figure 78A

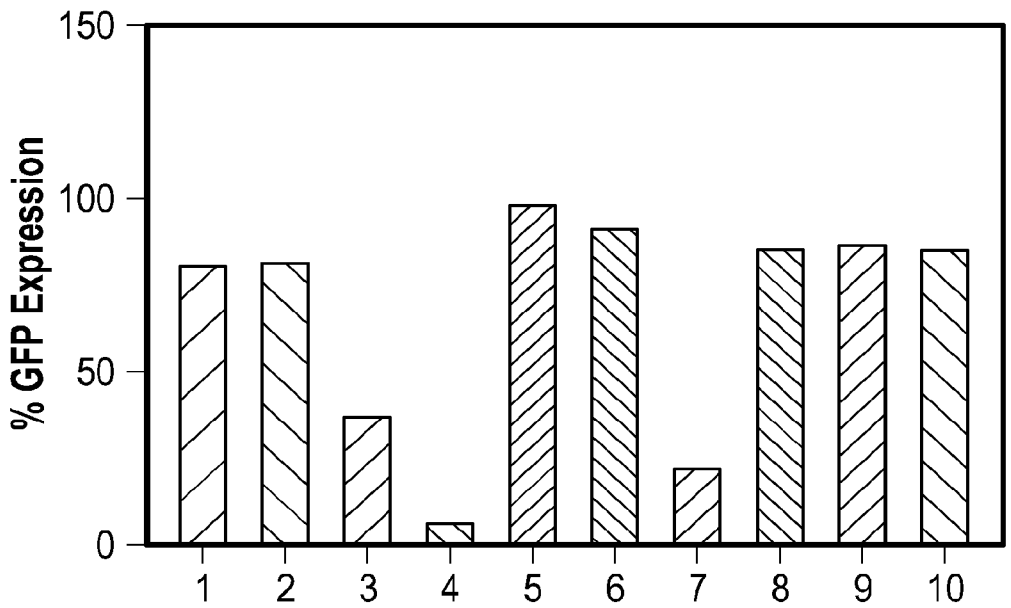

Figure 78B

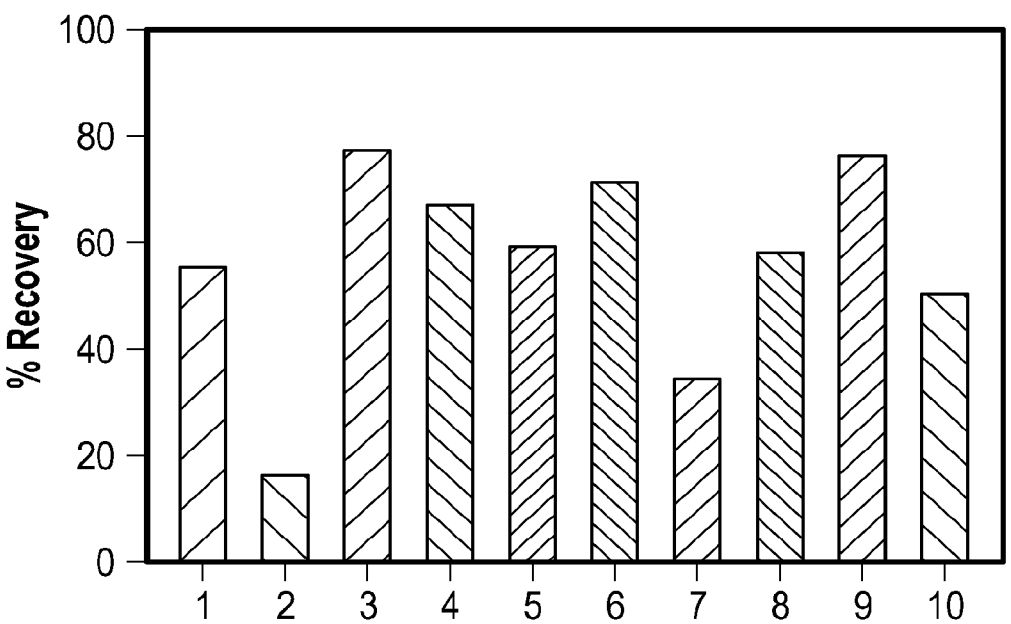

Figure 78C

Figure 79A

Figure 79B

Figure 79C

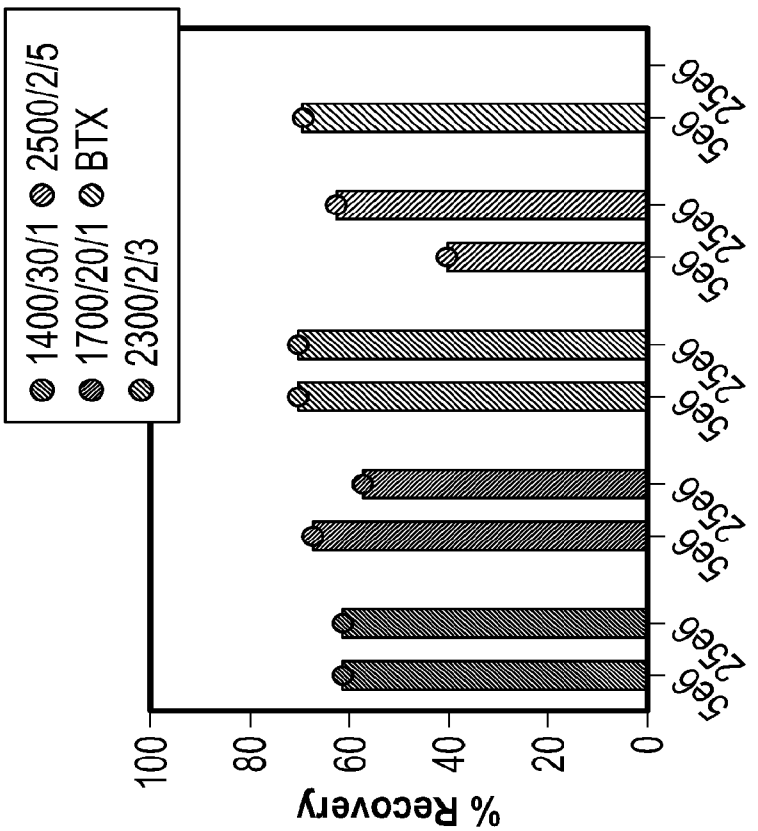

Figure 80B

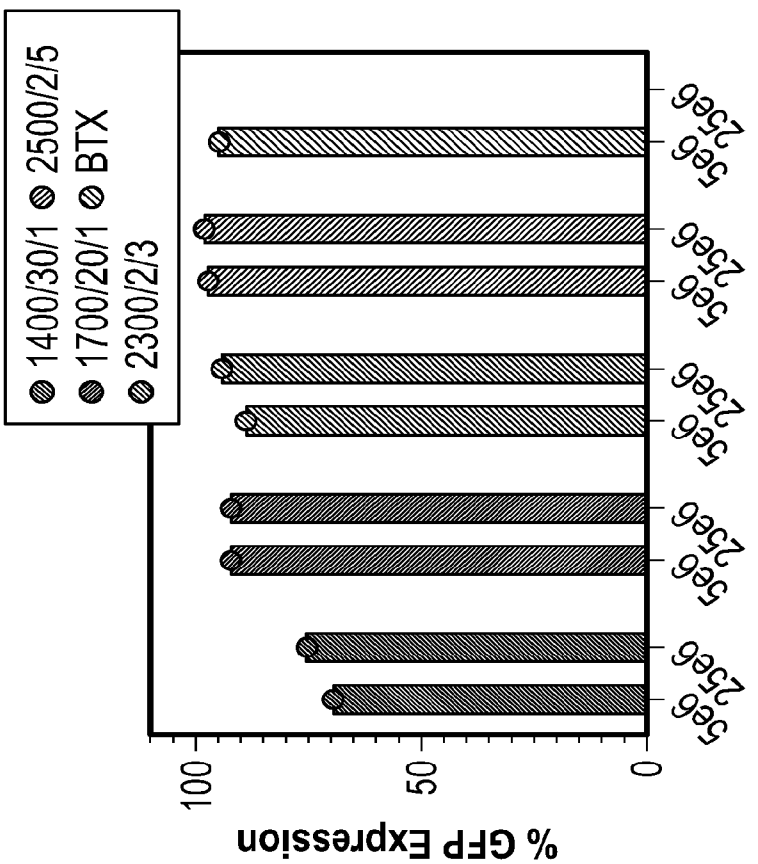

Figure 80A

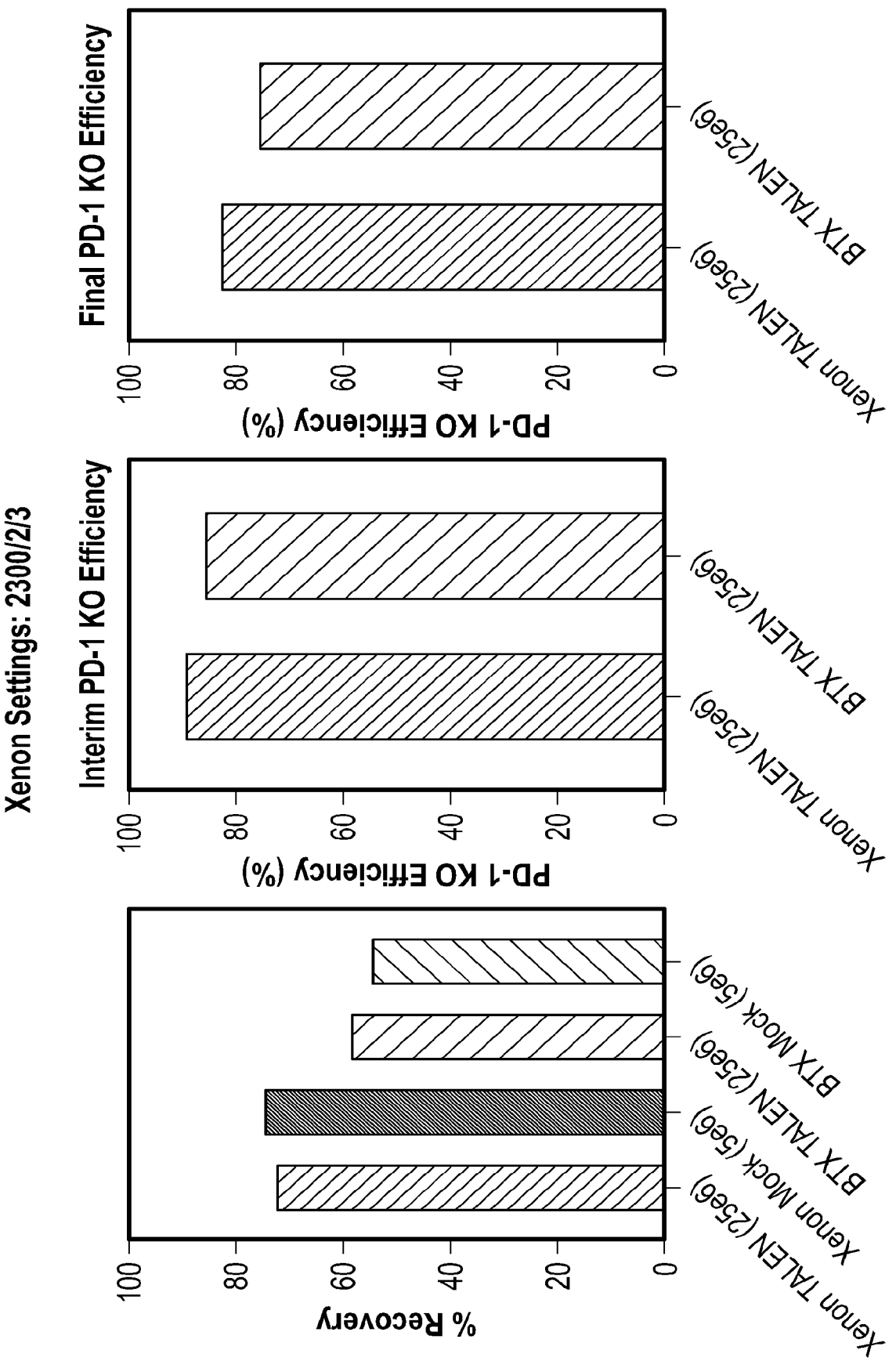

Figure 81A

Figure 81B

Figure 81C