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Canadian Intellectual Property Office

CA 3118634 A1 2020/05/14

(21) 3 118 634

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2019/11/04
(87) Date publication PCT/PCT Publication Date: 2020/05/14
(85) Entrée phase nationale/National Entry: 2021/05/03
(86) N° demande PCT/PCT Application No.: US 2019/059720
(87) N° publication PCT/PCT Publication No.: 2020/096989
(30) Priorités/Priorities: 2018/11/05 (US62/756,025); 2019/09/20 (US62/903,627)

(51) Cl.Int./Int.Cl. *A61K 35/17* (2015.01), *A61P 35/00* (2006.01), *C12N 5/0783* (2010.01)

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(54) Titre : TRAITEMENT DE PATIENTS SOUFFRANT DE NSCLC REFRACTAIRES A UN ANTICORPS ANTI-PD-1
(54) Title: TREATMENT OF NSCLC PATIENTS REFRACTORY FOR ANTI-PD-1 ANTIBODY

(57) Abrégé/Abstract:
The present invention provides improved and/or shortened processes and methods for preparing TILs in order to prepare therapeutic populations of TILs with increased therapeutic efficacy for the treatment of non-small cell lung carcinoma (NSCLC), wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 May 2020 (14.05.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/096989 A1

(51) International Patent Classification:
A61K 35/17 (2015.01) *C12N 5/0783* (2010.01)
A61P 35/00 (2006.01)

(21) International Application Number:
PCT/US2019/059720

(22) International Filing Date:
04 November 2019 (04.11.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/756,025 05 November 2018 (05.11.2018) US
62/903,627 20 September 2019 (20.09.2019) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2020/096989 A1

(54) Title: TREATMENT OF NSCLC PATIENTS REFRACTORY FOR ANTI-PD-1 ANTIBODY

(57) Abstract: The present invention provides improved and/or shortened processes and methods for preparing TILs in order to prepare therapeutic populations of TILs with increased therapeutic efficacy for the treatment of non-small cell lung carcinoma (NSCLC), wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

TREATMENT OF NSCLC PATIENTS REFRACTORY FOR ANTI-PD-1 ANTIBODY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 62/756,025, filed on November 5, 2018, and U.S. Provisional Patent Application No. 62/903,627, filed on September 20, 2019, which are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] Treatment of bulky, refractory cancers using adoptive 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. A large number of TILs are required for successful immunotherapy, and a robust and reliable process is needed for commercialization. This has been a challenge to achieve because of technical, logistical, and regulatory issues with cell expansion. IL-2-based TIL expansion followed by a "rapid expansion process" (REP) has become a preferred method for TIL expansion because of its speed and efficiency. Dudley, *et al.*, *Science* **2002,** *298,* 850-54; Dudley, *et al.*, *J. Clin. Oncol.* **2005,** *23,* 2346-57; Dudley, *et al.*, *J. Clin. Oncol.* **2008**, *26,* 5233-39; Riddell, *et al.*, *Science* **1992,** *257,* 238-41; Dudley, *et al.*, *J. Immunother.* **2003**, *26,* 332-42. REP can result in a 1,000-fold expansion of TILs over a 14-day period, although it requires a large excess (*e.g.*, 200-fold) of irradiated allogeneic peripheral blood mononuclear cells (PBMCs, also known as mononuclear cells (MNCs)), often from multiple donors, as feeder cells, as well as anti-CD3 antibody (OKT3) and high doses of IL-2. Dudley, *et al., J. Immunother.* **2003,** *26,* 332-42. TILs that have undergone an REP procedure have produced successful adoptive cell therapy following host immunosuppression in patients with melanoma. Current infusion acceptance parameters rely on readouts of the composition of TILs (*e.g.*, CD28, CD8, or CD4 positivity) and on fold expansion and viability of the REP product.

[0003] 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 anti-PD1 and as such have been severly 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 which can then be employed in the treatment of non-small cell lung carcinoma (NSCLC) patients whom are refractory to anti-PD-1 treatment.

BRIEF SUMMARY OF THE INVENTION

[0004] The present invention provides improved and/or shortened methods for expanding TILs and producing therapeutic populations of TILs for use in treatment of non-small cell lung carcinoma (NSCLC) patients whom are refractory to anti-PD-1 treatment.

[0005] The present invention provides a method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor infiltrating lymphocytes (TILs) comprising the steps of:

(a) obtaining and/or receiving a first population of TILs from surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells from a NSCLC tumor in a patient;

(c) contacting the tumor fragments with a first cell culture medium;

(d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

(e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

(f) harvesting the third population of TILs; and

(g) administering a therapeutically effective portion of the third population of TILs to a patient with the NSCLC;

wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

[0006] In some embodiments, "obtaining" indicates the TILs employed in the method and/or process can be derived directly from the sample (including from a surgical resection, needle biopsy, core biopsy, small biopsy, or other sample) as part of the method and/or process steps. In some embodiments, "receiving" indicates the TILs employed in the method and/or process can be derived indirectly from the sample (including from a surgical resection, needle biopsy, core biopsy, small biopsy, or other sample) and then employed in the method and/or process, (for example, where step (a) begins will TILs that have already been derived from the sample by a separate process not included in part (a), such TILs could be refered to as "received").

[0007] In some embodiments, the first population of TILs comprises a multilesional sampling method.

[0008] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 and/or anti-PD-L2 antibody.

[0009] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

[0010] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent.

[0011] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

[0012] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

[0013] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

[0014] In some embodiments, the refractory NSCLC has low expression of PD-L1.

[0015] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

[0016] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

[0017] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

[0018] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

[0019] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

[0020] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

[0021] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

[0022] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

[0023] In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

[0024] In some embodiments, the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

[0025] In some embodiments, the refractory NSCLC is refractory to an anti-PD-1 antibody or an anti-PD-L1 selected from the group consisting of nivolumab, pembrolizumab, ipilimumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

[0026] In some embodiments, the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

[0027] In some embodiments, the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

[0028] In some embodiments, the refractory NSCLC is refractory to ipilimumab or a biosimilar thereof.

[0029] In some embodiments, the refractory NSCLC is refractory to ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

[0030] In some embodiments, the refractory NSCLC is refractory to ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

[0031] In some embodiments, the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

[0032] In some embodiments, the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

[0033] In some embodiments, the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

[0034] In some embodiments, the initial expansion is performed over a period of 21 days or less.

[0035] In some embodiments, the initial expansion is performed over a period of 14 days or less.

[0036] In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

[0037] In some embodiments, 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 in the second cell culture medium.

[0038] In some embodiments, the initial expansion is performed using a gas permeable container.

[0039] In some embodiments, the rapid expansion is performed using a gas permeable container.

[0040] 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.

[0041] In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

[0042] In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

[0043] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m^2/day for two days followed by administration of fludarabine at a dose of 25 mg/m^2/day for five days.

[0044] In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

[0045] In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

[0046] In some embodiments, the invention provides a method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor infiltrating lymphocytes (TILs) comprising the steps of:

(a) resecting a tumor from a patient, the tumor comprising a first population of TILs;

(b) fragmenting the tumor into tumor fragments;

(c) contacting the tumor fragments with a first cell culture medium;

(d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

(e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

(f) harvesting the third population of TILs; and

(g) administering a therapeutically effective portion of the third population of TILs to a

patient with the cancer;

wherein the cancer is refractory to treatment with an anti-PD-1 antibody.

[0047] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

[0048] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

[0049] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent.

[0050] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

[0051] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

[0052] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

[0053] In some embodiments, the refractory NSCLC has low expression of PD-L1.

[0054] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

[0055] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

[0056] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

[0057] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

[0058] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

[0059] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

[0060] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

[0061] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

[0062] In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

[0063] In some embodiments, the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

[0064] In some embodiments, the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

[0065] In some embodiments, the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

[0066] In some embodiments, the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

[0067] In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

[0068] In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

[0069] In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

[0070] In some embodiments, the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

[0071] In some embodiments, the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

[0072] In some embodiments, the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

[0073] In some embodiments, the initial expansion is performed over a period of 21 days or less.

[0074] In some embodiments, the initial expansion is performed over a period of 14 days or less.

[0075] In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

[0076] In some embodiments, 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 in the second cell culture medium.

[0077] In some embodiments, the initial expansion is performed using a gas permeable container.

[0078] In some embodiments, the rapid expansion is performed using a gas permeable container.

[0079] 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.

[0080] In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

[0081] In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

[0082] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m^2/day for two days followed by administration of fludarabine at a dose of 25 mg/m^2/day for five days.

[0083] In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

[0084] In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

[0085] In some embodiments, the invention provides a method for treating a subject with non-small cell lung carcinoma (NSCLC), wherein the cancer is refractory to treatment with an anti-PD-1 antibody, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

(a) obtaining and/or receiving a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject 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 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-11~~3-14~~ days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-11~~7-14~~ days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;

(e) harvesting therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system; and

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

(g) cryopreserving the infusion bag comprising the harvested TIL population from step (f) using a cryopreservation process; and

(h) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

[0086] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

[0087] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

[0088] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent.

[0089] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

[0090] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

[0091] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

[0092] In some embodiments, the refractory NSCLC has low expression of PD-L1.

[0093] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

[0094] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

[0095] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

[0096] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1.

[0097] In some embodiments, the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

[0098] In some embodiments, the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

[0099] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

[00100] In some embodiments, the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

[00101] In some embodiments, bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

[00102] In some embodiments, the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

[00103] In some embodiments, the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

[00104] In some embodiments, the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

[00105] In some embodiments, the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

[00106] In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

[00107] In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

[00108] In some embodiments, the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

[00109] In some embodiments, the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

[00110] In some embodiments, the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

[00111] In some embodiments, the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

[00112] In some embodiments, the initial expansion is performed over a period of 21 days or less.

[00113] In some embodiments, the initial expansion is performed over a period of 14 days or less.

[00114] In some embodiments, the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

[00115] In some embodiments, 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 in the second cell culture medium.

[00116] In some embodiments, the initial expansion is performed using a gas permeable container.

[00117] In some embodiments, the rapid expansion is performed using a gas permeable container.

[00118] 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.

[00119] In some embodiments, the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

[00120] In some embodiments, the method further comprises the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

[00121] In some embodiments, the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m^2/day for two days followed by administration of fludarabine at a dose of 25 mg/m^2/day for five days.

[00122] In some embodiments, the method further comprises the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

[00123] In some embodiments, the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

[00124] In some embodiments, the NSCLC is refractory to a combination treatment comprising an anti-PD-1 and chemotherapeutic agent.

[00125] In some embodiments, the anti-PD-1 or the anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

[00126] In some embodiments, the antiPD-1 is pembrolizumab.

[00127] In some embodiments, the chemotherapeutic agent(s) is a platinum doublet chemotherapeutic agent.

[00128] In some embodiments, the platinum doublet therapy comprises:

i) a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin,

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

[00129] In some embodiments, the chemotherapeutic agent is in combination with pemetrexed.

[00130] In some embodiments, the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, and cisplatin.

[00131] In some embodiments, the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, cisplatin, nivolumab, and ipilimumab.

BRIEF DESCRIPTION OF THE DRAWINGS

[00132] Figure 1: Exemplary Process 2A chart providing an overview of Steps A through F.

[00133] Figure 2: Process Flow Chart of Process 2A.

[00134] Figure 3: Shows a diagram of an embodiment of a cryopreserved TIL exemplary manufacturing process (~22 days).

[00135] Figure 4: Shows a diagram of an embodiment of process 2A, a 22-day process for TIL manufacturing.

[00136] Figure 5: Comparison table of Steps A through F from exemplary embodiments of process 1C and process 2A.

[00137] Figure 6: Detailed comparison of an embodiment of process 1C and an embodiment of process 2A.

[00138] Figure 7: Exemplary GEN 3 type process for NSCLC tumors.

[00139] Figure 8A-8B: 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 Gen3 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.

[00140] Figure 9: Provides an experimental flow chart for comparability between GEN 2 (process 2A) versus GEN 3.

[00141] Figure 10A-10C: A) L4054 - Phenotypic characterization on TIL product on Gen 2 and Gen 3 process. **B)** L4055-Phenotypic characterization on TIL product on Gen 2 and Gen 3 process. **C)** M1085T-Phenotypic characterization on TIL product on Gen 2 and Gen 3 process.

[00142] Figure 11A-11C: **A)** L4054 – Memory markers analysis on TIL product from the Gen 2 and Gen 3 processes. **B)** L4055 – Memory markers analysis on TIL product from the

Gen 2 and Gen 3 processes. **C)** M1085T- Memory markers analysis on TIL product from the Gen 2 and Gen 3 processes.

[00143] Figure 12: L4054 Activation and exhaustion markers **(A)** Gated on CD4+, **(B)** Gated on CD8+.

[00144] Figure 13: L4055 Activation and exhaustion markers **(A)** Gated on CD4+, **(B)** Gated on CD8+.

[00145] Figure 14: IFNγ production (pg/mL): (A) L4054, (B) L4055, and (C) M1085T for the Gen 2 and Gen 3 processes: Each bar represented here is mean + SEM for IFNγ levels of stimulated, unstimulated, and media control. Optical density measured at 450 nm.

[00146] Figure 15: ELISA analysis of IL-2 concentration in cell culture supernatant: **(A) L4054** and **(B) L4055**. Each bar represented here is mean + SEM for IL-2 levels on spent media. Optical density measured at 450 nm.

[00147] Figure 16: Quantification of glucose and lactate (g/L) in spent media: (A) Glucose and (B) Lactate: In the two tumor lines, and in both processes, a decrease in glucose was observed throughout the REP expansion. Conversely, as expected, an increase in lactate was observed. Both the decrease in glucose and the increase in lactate were comparable between the Gen 2 and Gen 3 processes.

[00148] Figure 17: A) Quantification of L-glutamine in spent media for L4054 and L4055. **B)** Quantification of Glutamax in spent media for L4054 and L4055. C) Quantification of ammonia in spent media for L4054 and L4055.

[00149] Figure 18: Telomere length analysis. The relative telomere length (RTL) value indicates that the average telomere fluorescence per chromosome/genome in Gen 2 and Gen 3 process of the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

[00150] Figure 19: Unique CDR3 sequence analysis for TIL final product on L4054 and L4055 under Gen 2 and Gen 3 process. Columns show the number of unique TCR B clonotypes identified from 1×10^6 cells collected on Harvest Day Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16). Gen 3 shows higher clonal diversity compared to Gen 2 based on the number of unique peptide CDRs within the sample.

[00151] Figure 20: Frequency of unique CDR3 sequences on L4054 IL harvested final cell product (Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16)).

[00152] Figure 21: Frequency of unique CDR3 sequences on L4055 TIL harvested final cell product (Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16)).

[00153] Figure 22: Diversity Index for TIL final product on L4054 and L4055 under Gen 2 and Gen 3 process. Shanon entropy diversity index is a more reliable and common metric for comparison. Gen 3 L4054 and L4055 showed a slightly higher diversity than Gen 2.

[00154] Figure 23: Raw data for cell counts Day 7-Gen 3 REP initiation presented in Table 45 (see Example 8 below).

[00155] Figure 24: Raw data for cell counts Day 11-Gen 2 REP initiation and Gen 3 Scale Up presented in Table 45 (see Example 8 below).

[00156] Figure 25: Raw data for cell counts Day 16-Gen 2 Scale Up and Gen 3 Harvest (*e.g.*, day 16) presented in Table 46 (see Example 8 below).

[00157] Figure 26: Raw data for cell counts Day 22-Gen 2 Harvest (*e.g.*, day 22) presented in Table 46 (see Example 8 below). For L4054 Gen 2, post LOVO count was extrapolated to 4 flasks, because was the total number of the study. 1 flask was contaminated, and the extrapolation was done for total = 6.67E+10.

[00158] Figure 27: Raw data for flow cytometry results depicted in Figs. 10A, 10A, and 10B.

[00159] Figure 28: Raw data for flow cytometry results depicted in Figs. 10C and 10C.

[00160] Figure 29: Raw data for flow cytometry results depicted in Figs. 12 and 13.

[00161] Figure 30: Raw data for IFNγ production assay results for L4054 samples depicted in Fig. 14.

[00162] Figure 31: Raw data for IFNγ production assay results for L4055 samples depicted in Fig. 14.

[00163] Figure 32: Raw data for IFNγ production assay results for M1085T samples depicted in Fig. 14.

[00164] Figure 33: Raw data for IL-2 ELISA assay results depicted in Fig. 15.

[00165] Figure 34: Raw data for the metabolic substrate and metabolic analysis results presented in Figs. 16 and 17.

[00166] Figure 35: Raw data for the relative telomere length anaylsis results presented in Fig. 18.

[00167] Figure 36: Raw data for the unique CD3 sequence and clonal diversity analyses results presented in Figs. 19 and 22.

[00168] Figure 37: Shows a comparison between various Gen 2 (2A process) and the Gen 3.1 process embodiment.

[00169] Figure 38: Table describing various features of embodiments of the Gen 2, Gen 2.1 and Gen 3.0 process.

[00170] Figure 39: Overview of the media conditions for an embodiment of the Gen 3 process, referred to as Gen 3.1.

[00171] Figure 40: Table describing various features of embodiments of the Gen 2, Gen 2.1 and Gen 3.0 process.

[00172] Figure 41: Table comparing various features of embodiments of the Gen 2 and Gen 3.0 processes.

[00173] Figure 42: Table providing media uses in the various embodiments of the described expansion processes.

[00174] Figure 43: Phenotype comparison: Gen 3.0 and Gen 3.1 embodiments of the process showed comparable CD28, CD27 and CD57 expression.

[00175] Figure 44: Higher production of IFNγ on Gen 3 final product. IFNγ analysis (by ELISA) was assessed in the culture frozen supernatant to compared both processes. For each tumor overnight stimulation with coated anti -CD3 plate, using fresh TIL product on each Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 16). Each bar represents here are IFNγlevels of stimulated, unstimulated and media control.

[00176] Figure 45: Top: Unique CDR3 sequence analysis for TIL final product: Columns show the number of unique TCR B clonotypes identified from 1×10^6 cells collected on Gen 2 (*e.g.*, day 22) and Gen 3 process (*e.g.*, day 14-16). Gen 3 shows higher clonal diversity compared to Gen 2 based on the number of unique peptide CDRs within the sample. Bottom: Diversity Index for TIL final product: Shanon entropy diversity index is a more reliable a common metric for comparison. Gen 3 showed a slightly higher diversity than Gen 2.

[00177] Figure 46: 199 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 97.07% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

[00178] Figure 47: 1833 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 99.45% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

[00179] Figure 48: Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

[00180] Figure 49: Schematic of an exemplary embodiment of a method for expanding T cells from hematopoietic malignancies using Gen 3 expansion platform.

[00181] Figure 50: 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 VH and a VL 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.

[00182] Figure 51: Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

[00183] Figure 52: Provides a processs overview for an exemplary embodiment (Gen 3.1 Test) of the Gen 3.1 process (a 16 day process).

[00184] Figure 53: Provides data from TIL proliferation, average total viable cell counts per tumor fragment, percent viability at Harvest Day and total viable cell counts (TVC) at Harvest Day for exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). Gen 3.1 Test (which includes the addition of OKT-3 and feeders on Day 0) reached maximum capacity of the flask at harvest. If a maximum of 4 flasks are initiated on day 0, each TVC harvest should be multiplied by 4.

[00185] Figure 54: Bar graph depicting total viable cell count (TVC) and percent viability for exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test), a 16-day process.

[00186] Figure 55: Provides data showing that exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) yielded cells that showed comparable CD28, CD27 and CD57 expression.

[00187] Figure 56: Provides data showing TIL memory statuses were comparable across cells yielded by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, and Gen 3.1 Test). Memory statuses of REP TIL are depicted as follows: CD4+ or CD8+ TIL Memory subsets were divided into different memory subsets. Naïve (CD45RA+CD62L+), CM: Central memory (CD45RA-CD62L+), EM: Effector memory (CD45RA-CD62L-), TEMRA/TEFF: RA+ Effector memory/Effectors (CD45RA+CD62L+). Bar graph presented are percentage positive CD45+/-CD62L +/- when gated on CD4+ or CD8+.

[00188] Figure 57: Provides data showing TIL activation / exhaustion markers were comparable across cells yielded by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, and Gen 3.1 Test) when gated on CD4+. Activation and exhaustion of REP TIL were determined by multicolor flow cytometry. Harvested TIL samples were stained with flow cytometry antibodies (CD3-BUV395, PD-1-BV421, 2B4/CD244-PB, CD8-BB515, CD25-BUV563, BTLA-PE, KLRG1-PE-Dazzle 594, TIM-3-BV650, CD194/CCR4-APC, CD4-VioGreen, TIGIT-PerCP-eFluor 710, CD183-BV711, CD69-APC-R700, CD95-BUV737, CD127-PE-Cy7, CD103-BV786, LAG-3-APC-eFluor 780). Bar graph presented are percentage of CD4+ or CD8+ TIL of REP TIL.

[00189] Figure 58: Provides data showing TIL activation / exhaustion markers were comparable across cells yielded by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.0, Gen 3.1 Control and Gen 3.1) when gated on CD8+. Activation and exhaustion of REP TIL were determined by multicolor flow cytometry. TIL Harvested samples were stained with flow cytometry antibodies (CD3-BUV395, PD-1-BV421, 2B4/CD244-PB, CD8-BB515, CD25-BUV563, BTLA-PE, KLRG1-PE-Dazzle 594, TIM-3-BV650, CD194/CCR4-APC, CD4-VioGreen, TIGIT-PerCP-eFluor 710, CD183-BV711, CD69-APC-R700, CD95-BUV737, CD127-PE-Cy7, CD103-BV786, LAG-3-APC-eFluor 780). Bar graph presented are percentage of CD4+ or CD8+ TIL of REP TIL.

[00190] Figure 59: Provides data showing higher production of IFN-γ exhibited by Gen 3.1 final product. IFNγ analysis ELISA was assessed in the culture frozen supernatant to compare both processes. For each tumor overnight stimulation with coated anti -CD3 plate, using fresh TIL product on each Harvest day. Each bar represents here are IFN-γ levels of stimulated, unstimulated and media control.

[00191] Figure 60: Provides data showing that IL-2 concentration on supernatant were comparable across exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) using Standard media. Left panel: L4063- Gen 2 Standard Media. Right panel: L4064- CTS Optimizer Media. *ELISA performed with AIM V diluent

[00192] Figure 61: Provides data showing that metabolite concentrations were comparable on supernatant supernatants across exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). L4063 TILs were expanded in standard media. L4064 TILs were expanded in CTS Optimizer media.

[00193] Figure 62: Telomere length analysis on exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). Telomere length analysis for cells yielded by tumor identification numbers L4063 and L4064: the relative telomere length (RTL) value indicates the average telomere fluorescence per chromosome/genome in cells produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes over the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

[00194] Figure 63: Schematic of an exemplary embodiment of the Gen 3.1 Test (Gen 3.1 optimized) process (a 16-17 day process).

[00195] Figure 64: Schematic of an exemplary embodiment of the Gen 3 process (a 16-day process).

[00196] Figure 65A-65B: Comparison tables for exemplary Gen 2 and exemplary Gen 3 processes with exemplary differences highlighted.

[00197] Figure 66: Schematic of an exemplary embodiment of the Gen 3 process (a 16/17 day process) preparation timeline.

[00198] Figure 67: Schematic of an exemplary embodiment of the Gen 3 process (a 14-16 day process).

[00199] Figure 68: Summary of data from Day 16/17 of three engineering runs of an exemplary Gen 3 process embodiment.

[00200] Figure 69: Data regarding the extended phenotype of TIL: shown are the differentiation characteristics against TIL identity (ID) specifications for cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

[00201] Figure 70: Data regarding the extended phenotype of TIL expanded from lung tumors: shown are the differentiation characteristics against TIL identity (ID) specifications for cells produced by two process development (PD) runs of an exemplary Gen 3 process embodiment using lung tumor tissues.

[00202] Figure 71: Data regarding the extended phenotype (purity, identity and memory) of TIL expanded from ovarian tumors: shown are the purity, identity and memory phenotypic characteristics of cells expanded from ovarian tumors using exemplary Gen 2, Gen 3.1, and FR ER (Frozen tumor, Early REP) process embodiments; * indicates condition not tested; Y indicates sampling issue, low TVC count or non-viable cells on thawing.

[00203] Figure 72: Shown is the gating strategy for characterization of TIL (gating hierarchy is shown) and data regarding the extended phenotypic characteristics of cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

[00204] Figure 73: Shown is the gating strategy for characterization of TIL (gating hierarchy is shown) and data regarding the extended phenotypic characteristics of the CD4+ subpopulation and the CD8+ subpopulation of cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

[00205] Figure 74: Shown are data regarding Granzyme B ELISA analysis of cells produced by two engineering runs of an exemplary Gen 3 process embodiment.

[00206] Figure 75A-75B: Schematic of an exemplary embodiment of the Gen 3 process (a 16 day process).

[00207] Figure 76: Schematic of an exemplary embodiment of the Gen 3 process (a 16 day process).

[00208] Figure 77: Comparison of Gen 2, Gen 2.1 and an embodiment of the Gen 3 process (a 16 day process).

[00209] Figure 78: Comparison of Gen 2, Gen 2.1 and an embodiment of the Gen 3 process (a 16 day process).

[00210] Figure 79: Gen 3 embodiment components.

[00211] Figure 80: Gen 3 embodiment flow chart comparison (Gen 3.0, Gen 3.1 control, Gen 3.1 Test).

[00212] Figure 81: Total viable cell count and fold expansion are presented for exemplary Gen 3 embodiments (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and serum free cell culture media.

[00213] Figure 82: % viability scores upon reactivation, culture scale up and TIL harvest are presented for exemplary Gen 3 embodiments (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and serum free cell culture media.

[00214] Figure 83: Presented is phenotypic characterization of final TIL product produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00215] Figure 84: Presented is memory marker analysis of TIL product produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00216] Figure 85: Presented are activation and exhaustion markers of TIL produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media followed by CD4+ gated cell sorting.

[00217] Figure 86: Presented are activation and exhaustion markers of TIL produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media followed by CD8+ gated cell sorting.

[00218] Figure 87: Presented are IFN-γ production (pg/mL) scores for final TIL product produced by processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00219] Figure 88: Presented is IL-2 concentration (pg/mL) analysis of spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and

L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00220] Figure 89: Presented is concentration of glucose (g/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00221] Figure 90: Presented is concentration of lactate (g/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00222] Figure 91: Presented is concentration of glutamine (mmol/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00223] Figure 92: Presented is concentration of glutamax (mmol/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064 tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media.

[00224] Figure 93: Presented is concentration of ammonia (mmol/L) in spent media (collected upon reactivation, culture scale up and TIL harvest) from processing L4063 and L4064tumor samples in exemplary Gen 3 processes (Gen 3.0, Gen 3.1 Control and Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media. Telomere length analysis on exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test). Telomere length analysis for cells yielded by tumor identification numbers L4063 and L4064: the relative telomere length (RTL) value indicates the average telomere fluorescence per chromosome/genome in cells produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes over the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

[00225] Figure 94: Telomere length analysis on TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media. Telomere length analysis for cells yielded by tumor identification numbers L4063 and L4064: the relative telomere length (RTL) value

indicates the average telomere fluorescence per chromosome/genome in cells produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes over the telomere fluorescence per chromosome/genome in the control cells line (1301 Leukemia cell line) using DAKO kit.

[00226] Figure 95: TCR Vβ repertoire summary for TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) using standard cell culture media and CTS serum free cell culture media. Described is the clonality of TIL for final TIL product yielded by tumor identification numbers L4063 and L4064 produced by the Gen 3.0, Gen 3.1 Control and Gen 3.1 Test processes as measured by the TCR Vβ repertoire of unique CDR3 sequences.

[00227] Figure 96: Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to frequency of unique CDR3 sequences in TIL harvested product from processing of L4063 tumor samples.

[00228] Figure 97: Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to percentage shared unique CDR3 sequences in TIL harvested cell product from processing of L4063 tumor samples: 975 sequences are shared between Gen 3.0 and Gen 3.1 Test final product, equivalent to 88% of top 80% of unique CDR3 sequences from Gen 3.0 shared with Gen 3.1 Test final product.

[00229] Figure 98: Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to percentage shared unique CDR3 sequences in TIL harvested cell product for from processing of L4064 tumor samples: 2163 sequences are shared between Gen 3.0 and Gen 3.1 Test final product, equivalent to 87% of top 80% of unique CDR3 sequences from Gen 3.0 shared with Gen 3.1 Test final product.

[00230] Figure 99: Comparison of TIL produced by exemplary embodiments of the Gen 3 process (Gen 3.0, Gen 3.1 Control, Gen 3.1 Test) with respect to frequency of unique CDR3 sequences in TIL harvested product from processing of L4064 tumor samples.

[00231] Figure 100: Shown are the components of an exemplary embodiment of the Gen 3 process (Gen 3-Optimized, a 16-17 day process).

[00232] Figure 101: Acceptance criteria table.

[00233] Figure 102: Cell counts reactivation Day.

[00234] Figure 103: Cell counts Scale Up Day.

[00235] Figure 104: Cell counts Harvest L4063.

[00236] Figure 105: Cell counts Harvest L4064.

[00237] Figure 106: Flow data.

[00238] Figure 107: Flow data.

[00239] Figure 108: Flow data.

[00240] Figure 109: Flow data.

[00241] Figure 110: IFN-γ production Data Figure 7-L4063.

[00242] Figure 111: Data IFN-γ production Figure 7-L4064.

[00243] Figure 112: ELISA analysis of IL-2 concentration data.

[00244] Figure 113: Metabolic data summary table.

[00245] Figure 114: Summary data.

[00246] Figure 115: Summary data.

[00247] Figure 116: Shannon diversity index.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

[00248] SEQ ID NO:1 is the amino acid sequence of the heavy chain of muromonab.

[00249] SEQ ID NO:2 is the amino acid sequence of the light chain of muromonab.

[00250] SEQ ID NO:3 is the amino acid sequence of a recombinant human IL-2 protein.

[00251] SEQ ID NO:4 is the amino acid sequence of aldesleukin.

[00252] SEQ ID NO:5 is the amino acid sequence of a recombinant human IL-4 protein.

[00253] SEQ ID NO:6 is the amino acid sequence of a recombinant human IL-7 protein.

[00254] SEQ ID NO:7 is the amino acid sequence of a recombinant human IL-15 protein.

[00255] SEQ ID NO:8 is the amino acid sequence of a recombinant human IL-21 protein.

[00256] SEQ ID NO:9 is the amino acid sequence of human 4-1BB.

[00257] SEQ ID NO:10 is the amino acid sequence of murine 4-1BB.

[00258] SEQ ID NO:11 is the heavy chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00259] SEQ ID NO:12 is the light chain for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00260] SEQ ID NO:13 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00261] SEQ ID NO:14 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00262] SEQ ID NO:15 is the heavy chain CDRl for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00263] SEQ ID NO:16 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00264] SEQ ID NO:17 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00265] SEQ ID NO:18 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00266] SEQ ID NO:19 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00267] SEQ ID NO:20 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody utomilumab (PF-05082566).

[00268] SEQ ID NO:21 is the heavy chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00269] SEQ ID NO:22 is the light chain for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00270] SEQ ID NO:23 is the heavy chain variable region (VH) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00271] SEQ ID NO:24 is the light chain variable region (VL) for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00272] SEQ ID NO:25 is the heavy chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00273] SEQ ID NO:26 is the heavy chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00274] SEQ ID NO:27 is the heavy chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00275] SEQ ID NO:28 is the light chain CDR1 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00276] SEQ ID NO:29 is the light chain CDR2 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00277] SEQ ID NO:30 is the light chain CDR3 for the 4-1BB agonist monoclonal antibody urelumab (BMS-663513).

[00278] SEQ ID NO:31 is an Fc domain for a TNFRSF agonist fusion protein.

[00279] SEQ ID NO:32 is a linker for a TNFRSF agonist fusion protein.

[00280] SEQ ID NO:33 is a linker for a TNFRSF agonist fusion protein.

[00281] SEQ ID NO:34 is a linker for a TNFRSF agonist fusion protein.

[00282] SEQ ID NO:35 is a linker for a TNFRSF agonist fusion protein.

[00283] SEQ ID NO:36 is a linker for a TNFRSF agonist fusion protein.

[00284] SEQ ID NO:37 is a linker for a TNFRSF agonist fusion protein.

[00285] SEQ ID NO:38 is a linker for a TNFRSF agonist fusion protein.

[00286] SEQ ID NO:39 is a linker for a TNFRSF agonist fusion protein.

[00287] SEQ ID NO:40 is a linker for a TNFRSF agonist fusion protein.

[00288] SEQ ID NO:41 is a linker for a TNFRSF agonist fusion protein.

[00289] SEQ ID NO:42 is an Fc domain for a TNFRSF agonist fusion protein.

[00290] SEQ ID NO:43 is a linker for a TNFRSF agonist fusion protein.

[00291] SEQ ID NO:44 is a linker for a TNFRSF agonist fusion protein.

[00292] SEQ ID NO:45 is a linker for a TNFRSF agonist fusion protein.

[00293] SEQ ID NO:46 is a 4-1BB ligand (4-1BBL) amino acid sequence.

[00294] SEQ ID NO:47 is a soluble portion of 4-1BBL polypeptide.

[00295] SEQ ID NO:48 is a heavy chain variable region (VH) for the 4-1BB agonist antibody 4B4-1-1 version 1.

[00296] SEQ ID NO:49 is a light chain variable region (VL) for the 4-1BB agonist antibody 4B4-1-1 version 1.

[00297] SEQ ID NO:50 is a heavy chain variable region (VH) for the 4-1BB agonist antibody 4B4-1-1 version 2.

[00298] SEQ ID NO:51 is a light chain variable region (VL) for the 4-1BB agonist antibody 4B4-1-1 version 2.

[00299] SEQ ID NO:52 is a heavy chain variable region (VH) for the 4-1BB agonist antibody H39E3-2.

[00300] SEQ ID NO:53 is a light chain variable region (VL) for the 4-1BB agonist antibody H39E3-2.

[00301] SEQ ID NO:54 is the amino acid sequence of human OX40.

[00302] SEQ ID NO:55 is the amino acid sequence of murine OX40.

[00303] SEQ ID NO:56 is the heavy chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00304] SEQ ID NO:57 is the light chain for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00305] SEQ ID NO:58 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00306] SEQ ID NO:59 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00307] SEQ ID NO:60 is the heavy chain CDRl for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00308] SEQ ID NO:61 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00309] SEQ ID NO:62 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00310] SEQ ID NO:63 is the light chain CDR1 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00311] SEQ ID NO:64 is the light chain CDR2 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00312] SEQ ID NO:65 is the light chain CDR3 for the OX40 agonist monoclonal antibody tavolixizumab (MEDI-0562).

[00313] SEQ ID NO:66 is the heavy chain for the OX40 agonist monoclonal antibody 11D4.

[00314] SEQ ID NO:67 is the light chain for the OX40 agonist monoclonal antibody 11D4.

[00315] SEQ ID NO:68 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 11D4.

[00316] SEQ ID NO:69 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 11D4.

[00317] SEQ ID NO:70 is the heavy chain CDRl for the OX40 agonist monoclonal antibody 11D4.

[00318] SEQ ID NO:71 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

[00319] SEQ ID NO:72 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

[00320] SEQ ID NO:73 is the light chain CDR1 for the OX40 agonist monoclonal antibody 11D4.

[00321] SEQ ID NO:74 is the light chain CDR2 for the OX40 agonist monoclonal antibody 11D4.

[00322] SEQ ID NO:75 is the light chain CDR3 for the OX40 agonist monoclonal antibody 11D4.

[00323] SEQ ID NO:76 is the heavy chain for the OX40 agonist monoclonal antibody 18D8.

[00324] SEQ ID NO:77 is the light chain for the OX40 agonist monoclonal antibody 18D8.

[00325] SEQ ID NO:78 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 18D8.

[00326] SEQ ID NO:79 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 18D8.

[00327] SEQ ID NO:80 is the heavy chain CDRl for the OX40 agonist monoclonal antibody 18D8.

[00328] SEQ ID NO:81 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

[00329] SEQ ID NO:82 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

[00330] SEQ ID NO:83 is the light chain CDR1 for the OX40 agonist monoclonal antibody 18D8.

[00331] SEQ ID NO:84 is the light chain CDR2 for the OX40 agonist monoclonal antibody 18D8.

[00332] SEQ ID NO:85 is the light chain CDR3 for the OX40 agonist monoclonal antibody 18D8.

[00333] SEQ ID NO:86 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody Hu119-122.

[00334] SEQ ID NO:87 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody Hu119-122.

[00335] SEQ ID NO:88 is the heavy chain CDRl for the OX40 agonist monoclonal antibody Hu119-122.

[00336] SEQ ID NO:89 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

[00337] SEQ ID NO:90 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

[00338] SEQ ID NO:91 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu119-122.

[00339] SEQ ID NO:92 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu119-122.

[00340] SEQ ID NO:93 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu119-122.

[00341] SEQ ID NO:94 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody Hu106-222.

[00342] SEQ ID NO:95 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody Hu106-222.

[00343] SEQ ID NO:96 is the heavy chain CDRl for the OX40 agonist monoclonal antibody Hu106-222.

[00344] SEQ ID NO:97 is the heavy chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

[00345] SEQ ID NO:98 is the heavy chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

[00346] SEQ ID NO:99 is the light chain CDR1 for the OX40 agonist monoclonal antibody Hu106-222.

[00347] SEQ ID NO:100 is the light chain CDR2 for the OX40 agonist monoclonal antibody Hu106-222.

[00348] SEQ ID NO:101 is the light chain CDR3 for the OX40 agonist monoclonal antibody Hu106-222.

[00349] SEQ ID NO:102 is an OX40 ligand (OX40L) amino acid sequence.

[00350] SEQ ID NO:103 is a soluble portion of OX40L polypeptide.

[00351] SEQ ID NO:104 is an alternative soluble portion of OX40L polypeptide.

[00352] SEQ ID NO:105 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 008.

[00353] SEQ ID NO:106 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 008.

[00354] SEQ ID NO:107 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 011.

[00355] SEQ ID NO:108 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 011.

[00356] SEQ ID NO:109 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 021.

[00357] SEQ ID NO:110 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 021.

[00358] SEQ ID NO:111 is the heavy chain variable region (VH) for the OX40 agonist monoclonal antibody 023.

[00359] SEQ ID NO:112 is the light chain variable region (VL) for the OX40 agonist monoclonal antibody 023.

[00360] SEQ ID NO:113 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.

[00361] SEQ ID NO:114 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

[00362] SEQ ID NO:115 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.

[00363] SEQ ID NO:116 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

[00364] SEQ ID NO:117 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

[00365] SEQ ID NO:118 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

[00366] SEQ ID NO:119 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

[00367] SEQ ID NO:120 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

[00368] SEQ ID NO:121 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

[00369] SEQ ID NO:122 is the heavy chain variable region (VH) for a humanized OX40 agonist monoclonal antibody.

[00370] SEQ ID NO:123 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

[00371] SEQ ID NO:124 is the light chain variable region (VL) for a humanized OX40 agonist monoclonal antibody.

[00372] SEQ ID NO:125 is the heavy chain variable region (VH) for an OX40 agonist monoclonal antibody.

[00373] SEQ ID NO:126 is the light chain variable region (VL) for an OX40 agonist monoclonal antibody.

[00374] SEQ ID NO:127-462 are currently not assigned.

[00375] SEQ ID NO:463 is the heavy chain amino acid sequence of the PD-1 inhibitor nivolumab.

[00376] SEQ ID NO:464 is the light chain amino acid sequence of the PD-1 inhibitor nivolumab.

[00377] SEQ ID NO:465 is the heavy chain variable region (V_H) amino acid sequence of the PD-1 inhibitor nivolumab.

[00378] SEQ ID NO:466 is the light chain variable region (V_L) amino acid sequence of the PD-1 inhibitor nivolumab.

[00379] SEQ ID NO:467 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

[00380] SEQ ID NO:468 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

[00381] SEQ ID NO:469 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

[00382] SEQ ID NO:470 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor nivolumab.

[00383] SEQ ID NO:471 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor nivolumab.

[00384] SEQ ID NO:472 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor nivolumab.

[00385] SEQ ID NO:473 is the heavy chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00386] SEQ ID NO:474 is the light chain amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00387] SEQ ID NO:475 is the heavy chain variable region (V_H) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00388] SEQ ID NO:476 is the light chain variable region (V_L) amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00389] SEQ ID NO:477 is the heavy chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00390] SEQ ID NO:478 is the heavy chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00391] SEQ ID NO:479 is the heavy chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00392] SEQ ID NO:480 is the light chain CDR1 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00393] SEQ ID NO:481 is the light chain CDR2 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00394] SEQ ID NO:482 is the light chain CDR3 amino acid sequence of the PD-1 inhibitor pembrolizumab.

[00395] SEQ ID NO:483 is the heavy chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00396] SEQ ID NO:484 is the light chain amino acid sequence of the PD-L1 inhibitor durvalumab.

[00397] SEQ ID NO:485 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00398] SEQ ID NO:486 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor durvalumab.

[00399] SEQ ID NO:487 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00400] SEQ ID NO:488 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00401] SEQ ID NO:489 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00402] SEQ ID NO:490 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00403] SEQ ID NO:491 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00404] SEQ ID NO:492 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor durvalumab.

[00405] SEQ ID NO:493 is the heavy chain amino acid sequence of the PD-L1 inhibitor avelumab.

[00406] SEQ ID NO:494 is the light chain amino acid sequence of the PD-L1 inhibitor avelumab.

[00407] SEQ ID NO:495 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor avelumab.

[00408] SEQ ID NO:496 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor avelumab.

[00409] SEQ ID NO:497 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

[00410] SEQ ID NO:498 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

[00411] SEQ ID NO:499 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

[00412] SEQ ID NO:500 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor avelumab.

[00413] SEQ ID NO:501 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor avelumab.

[00414] SEQ ID NO:502 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor avelumab.

[00415] SEQ ID NO:503 is the heavy chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00416] SEQ ID NO:504 is the light chain amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00417] SEQ ID NO:505 is the heavy chain variable region (V_H) amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00418] SEQ ID NO:506 is the light chain variable region (V_L) amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00419] SEQ ID NO:507 is the heavy chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00420] SEQ ID NO:508 is the heavy chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00421] SEQ ID NO:509 is the heavy chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00422] SEQ ID NO:510 is the light chain CDR1 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00423] SEQ ID NO:511 is the light chain CDR2 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00424] SEQ ID NO:512 is the light chain CDR3 amino acid sequence of the PD-L1 inhibitor atezolizumab.

[00425]

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[00426] Adoptive cell therapy utilizing TILs cultured *ex vivo* by the Rapid Expansion Protocol (REP) has produced successful adoptive cell therapy following host immunosuppression in patients with cancer such as melanoma. Current infusion acceptance parameters rely on readouts of the composition of TILs (*e.g.*, CD28, CD8, or CD4 positivity) and on the numerical folds of expansion and viability of the REP product.

[00427] Current REP protocols give little insight into the health of the TIL that will be infused into the patient. T cells undergo a profound metabolic shift during the course of their maturation from naïve to effector T cells (see Chang, *et al.*, *Nat. Immunol.* **2016,** *17,* 364, hereby expressly incorporated in its entirety, and in particular for the discussion and markers of anaerobic and aerobic metabolism). For example, naïve T cells rely on mitochondrial respiration to produce ATP, while mature, healthy effector T cells such as TIL are highly glycolytic, relying on aerobic glycolysis to provide the bioenergetics substrates they require for proliferation, migration, activation, and anti-tumor efficacy.

[00428] 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 anti-PD1 and as such have been severly 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 which can then be employed in the treatment of non-small cell lung carcinoma (NSCLC) patients whom are refractory to anti-PD-1 treatment.

II. Definitions

[00429] 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.

[00430] 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.

[00431] The term "*in vivo*" refers to an event that takes place in a subject's body.

[00432] 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.

[00433] 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.

[00434] 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.

[00435]

[00436] 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.

[00437] 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.

[00438] 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.

[00439] 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.

[00440] 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.

[00441] 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.

[00442] 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.

[00443] 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.

[00444] 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.

[00445] 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.

[00446] 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.

[00447] 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+.

[00448] 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.

[00449] The term "OKT-3" (also referred to herein as "OKT3") refers to a monoclonal antibody or biosimilar or variant thereof, including human, humanized, chimeric, or murine antibodies, directed against the CD3 receptor in the T cell antigen receptor of mature T cells, and includes commercially-available forms such as OKT-3 (30 ng/mL, MACS GMP CD3 pure, Miltenyi Biotech, Inc., San Diego, CA, USA) and muromonab or variants, conservative amino acid substitutions, glycoforms, or biosimilars thereof. The amino acid sequences of the heavy and light chains of muromonab are given in Table 1 (SEQ ID NO:1 and SEQ ID NO:2). A hybridoma capable of producing OKT-3 is deposited with the American Type Culture Collection and assigned the ATCC accession number CRL 8001. A hybridoma capable of producing OKT-3 is also deposited with European Collection of Authenticated Cell Cultures (ECACC) and assigned Catalogue No. 86022706.

TABLE 1. Amino acid sequences of muromonab.

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

[00450] The term "IL-2" (also referred to herein as "IL2") refers to the T cell growth factor known as interleukin-2, and includes all forms of IL-2 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-2 is described, *e.g.*, in Nelson, *J. Immunol.* **2004,** *172,* 3983-88 and Malek, *Annu. Rev. Immunol.* **2008,** *26,* 453-79, the disclosures of which are incorporated by reference herein.

The amino acid sequence of recombinant human IL-2 suitable for use in the invention is given in Table 2 (SEQ ID NO:3). For example, the term IL-2 encompasses human, recombinant forms of IL-2 such as aldesleukin (PROLEUKIN, available commercially from multiple suppliers in 22 million IU per single use vials), as well as the form of recombinant IL-2 commercially supplied by CellGenix, Inc., Portsmouth, NH, USA (CELLGRO GMP) or ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-209-b) and other commercial equivalents from other vendors. Aldesleukin (des-alanyl-1, serine-125 human IL-2) is a nonglycosylated human recombinant form of IL-2 with a molecular weight of approximately 15 kDa. The amino acid sequence of aldesleukin suitable for use in the invention is given in Table 2 (SEQ ID NO:4). The term IL-2 also encompasses pegylated forms of IL-2, as described herein, including the pegylated IL2 prodrug NKTR-214, available from Nektar Therapeutics, South San Francisco, CA, USA. NKTR-214 and pegylated IL-2 suitable for use in the invention is described in U.S. Patent Application Publication No. US 2014/0328791 A1 and International Patent Application Publication No. WO 2012/065086 Al, 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 4902,502, 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.

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 EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN RWITFCQSII STLT	60 120 134
SEQ ID NO:4 Aldesleukin	PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW ITFSQSIIST LT	60 120 132
SEQ ID NO:5 recombinant human IL-4 (rhIL-4)	MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI MREKYSKCSS	60 120 130
SEQ ID NO:6 recombinant human IL-7 (rhIL-7)	MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH	60 120 153
SEQ ID NO:7 recombinant human IL-15 (rhIL-15)	MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS	60 115
SEQ ID NO:8 recombinant human IL-21 (rhIL-21)	MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ HLSSRTHGSE DS	60 120 132

[00451] 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:5).

[00452] The term "IL-7" (also referred to herein as "IL7") refers to a glycosylated tissue-derived cytokine known as interleukin 7, which may be obtained from stromal and epithelial cells, as well as from dendritic cells. Fry and Mackall, *Blood* **2002,** *99,* 3892-904. IL-7 can stimulate the development of T cells. IL-7 binds to the IL-7 receptor, a heterodimer consisting of IL-7 receptor alpha and common gamma chain receptor, which in a series of signals important for T cell development within the thymus and survival within the periphery. Recombinant human IL-7 suitable for use in the invention is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-254) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15 recombinant protein, Cat. No. Gibco PHC0071). The amino acid sequence of recombinant human IL-7 suitable for use in the invention is given in Table 2 (SEQ ID NO:6).

[00453] The term "IL-15" (also referred to herein as "IL15") refers to the T cell growth factor known as interleukin-15, and includes all forms of IL-2 including human and mammalian forms, conservative amino acid substitutions, glycoforms, biosimilars, and variants thereof. IL-15 is described, *e.g.*, in Fehniger and Caligiuri, *Blood* **2001,** *97,* 14-32, the disclosure of which is incorporated by reference herein. IL-15 shares β and γ signaling receptor subunits with IL-2. Recombinant human IL-15 is a single, non-glycosylated polypeptide chain containing 114 amino acids (and an N-terminal methionine) with a molecular mass of 12.8 kDa. Recombinant human IL-15 is commercially available from multiple suppliers, including ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA (Cat. No. CYT-230-b) and ThermoFisher Scientific, Inc., Waltham, MA, USA (human IL-15

recombinant protein, Cat. No. 34-8159-82). The amino acid sequence of recombinant human IL-15 suitable for use in the invention is given in Table 2 (SEQ ID NO:7).

[00454] 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:8).

[00455] When "an anti-tumor effective amount", "an 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. Tumor infiltrating lymphocytes (including in some cases, genetically modified cytotoxic lymphocytes) compositions may also be administered multiple times at these dosages. The tumor infiltrating lymphocytes (inlcuding in some cases, genetically) can be administered by using infusion techniques that are commonly known in immunotherapy (*see,* e.g., Rosenberg et al., *New Eng. J. of Med.* 319: 1676, 1988). 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.

[00456] 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), 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.

[00457] 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.

[00458] 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.

[00459] In an embodiment, the invention includes a method of treating a cancer with a population of TILs, wherein a patient is pre-treated with non-myeloablative chemotherapy prior to an infusion of TILs according to the invention. In some embodiments, the population of TILs may be provided wherein a patient is pre-treated with nonmyeloablative chemotherapy prior to an infusion of TILs according to the present invention. In an embodiment, the non-myeloablative chemotherapy is cyclophosphamide 60 mg/kg/d for 2 days (days 27 and 26 prior to TIL infusion) and fludarabine 25 mg/m2/d for 5 days (days 27 to 23 prior to TIL infusion). In an embodiment, after non-myeloablative chemotherapy and

TIL infusion (at day 0) according to the invention, the patient receives an intravenous infusion of IL-2 intravenously at 720,000 IU/kg every 8 hours to physiologic tolerance.

[00460] Experimental findings indicate that lymphodepletion prior to adoptive transfer of tumor-specific T lymphocytes plays a key role in enhancing treatment efficacy by eliminating regulatory T cells and competing elements of the immune system ("cytokine sinks"). Accordingly, some embodiments of the invention utilize a lymphodepletion step (sometimes also referred to as "immunosuppressive conditioning") on the patient prior to the introduction of the rTILs of the invention.

[00461] 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.

[00462] 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.

[00463] 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).

[00464] 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.

[00465] 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.

[00466] 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).

[00467] 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.

[00468] 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.

[00469] 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.

[00470] 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.

[00471] 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.

[00472] 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."

[00473] 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.

[00474] 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.

[00475] 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.

[00476] 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.

[00477] 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.

[00478] 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 an embodiment, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic nonhuman animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

[00479] 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*.

[00480] As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by the heavy chain constant region genes.

[00481] 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."

[00482] 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.

[00483] 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.

[00484] 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.

[00485] 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.

[00486] 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.

[00487] "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.

[00488] 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.

III. TIL Manufacturing Processes – 2A

[00489] An exemplary TIL process known as process 2A containing some of these features is depicted in Figure 2, and some of the advantages of this embodiment of the present invention over process 1C are described in Figures F and G. An embodiment of process 2A is shown Figure 1.

[00490] 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.

[00491] 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.

[00492] In some embodiments, the first expansion (including processes referred to as the preREP as well as processes shown in Figure 1 as Step A) is shortened to 3 to 14 days and the second expansion (including processes referred to as the REP as well as processes shown in Figure 1 as Step B) is 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) is shortened to 11 days and the second expansion (for example, an expansion as described in Step D in Figure 1) is shortened to 11 days. In some embodiments, the combination of the first expansion and second expansion (for example, expansions described as Step B and Step D in Figure 1) is shortened to 22 days, as discussed in detail below and in the examples and figures.

[00493] The "Step" Designations A, B, C, *etc.*, below are in reference to Figure 1 and in reference to certain embodiments described herein. The ordering of the Steps below and in Figure 1 is exemplary and any combination or order of steps, as well as additional steps, repetition of steps, and/or omission of steps is contemplated by the present application and the methods disclosed herein.

A. STEP A: Obtain Patient tumor sample

[00494] In general, TILs are initially obtained from a patient tumor sample ("primary TILs") and then expanded into a larger population for further manipulation as described herein, optionally cryopreserved, restimulated as outlined herein and optionally evaluated for phenotype and metabolic parameters as an indication of TIL health.

[00495] 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 cites 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).

[00496] 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.

[00497] 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 with the enzymes to form a tumor digest reaction mixture.

[00498] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00499] In some embodiments, the enxyme 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.

[00500] 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.

[00501] 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.

[00502] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml DNAse, and 1 mg/ml hyaluronidase.

[00503] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml DNAse, and 1 mg/ml hyaluronidase.

[00504] In general, the harvested cell suspension is called a "primary cell population" or a "freshly harvested" cell population.

[00505] In some embodiments, fragmentation includes physical fragmentation, including for example, dissection as well as digestion. In some embodiments, the fragmentation is physical fragmentation. In some embodiments, the fragmentation is dissection. In some embodiments, the fragmentation is by digestion. In some embodiments, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients. In an embodiment, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients.

[00506] In some embodiments, where the tumor is a solid tumor, the tumor undergoes physical fragmentation after the tumor sample is obtained in, for example, Step A (as provided in Figure 1). In some embodiments, the fragmentation occurs before cryopreservation. In some embodiments, the fragmentation occurs after cryopreservation. In some embodiments, the fragmentation occurs after obtaining the tumor and in the absence of any cryopreservation. In some embodiments, the tumor is fragmented and 10, 20, 30, 40 or more fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 30 or 40 fragments or pieces are placed in each container for the first expansion. In some embodiments, the tumor is fragmented and 40

fragments or pieces are placed in each container for the first expansion. In some embodiments, the multiple fragments comprise about 4 to about 50 fragments, wherein each fragment has a volume of about 27 mm^3. In some embodiments, the multiple fragments comprise about 30 to about 60 fragments with a total volume of about 1300 mm^3 to about 1500 mm^3. In some embodiments, the multiple fragments comprise about 50 fragments with a total volume of about 1350 mm^3. In some embodiments, the multiple fragments comprise about 50 fragments with a total mass of about 1 gram to about 1.5 grams. In some embodiments, the multiple fragments comprise about 4 fragments.

[00507] 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 x 1-4 mm x 1-4 mm. In some embodiments, the tumors are 1 mm x 1 mm x 1 mm. In some embodiments, the tumors are 2 mm x 2 mm x 2 mm. In some embodiments, the tumors are 3 mm x 3 mm x 3 mm. In some embodiments, the tumors are 4 mm x 4 mm x 4 mm.

[00508] 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.

[00509] 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.

[00510] 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.

[00511] 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.

B. STEP B: First Expansion

[00512] 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 Donia, at al., *Scandinavian Journal of Immunology,* 75:157–167 (2012); Dudley et al., *Clin Cancer Res,* 16:6122-6131 (2010); Huang et al., *J Immunother*, 28(3):258–267 (2005); Besser et al., *Clin Cancer Res*, 19(17):OF1-OF9 (2013); Besser et al., *J Immunother* 32:415–423 (2009); Robbins, et al., *J Immunol* 2004; 173:7125-7130; Shen et al., J Immunother, 30:123–129 (2007); Zhou, et al., *J Immunother*, 28:53–62 (2005); and Tran, et al., J Immunother, 31:742–751 (2008), all of which are incorporated herein by reference in their entireties.

[00513] The diverse antigen receptors of T and B lymphocytes are produced by somatic recombination of a limited, but large number of gene segments. These gene segments: V (variable), D (diversity), J (joining), and C (constant), determine the binding

specificity and downstream applications of immunoglobulins and T-cell receptors (TCRs). The present invention provides a method for generating TILs which exhibit and increase the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using other methods than those provide herein including for example, methods other than those embodied in Figure 1. In some embodiments, the TILs obtained by the present method exhibit an increase in the T-cell repertoire diversity as compared to freshly harvested TILs and/or TILs prepared using methods referred to as process 1C, as exemplified in Figure 5 and/or Figure 6. In some embodiments, the TILs obtained in the first expansion exhibit an increase in the T-cell repertoire diversity. In some embodiments, the increase in diversity is an increase in the immunoglobulin diversity and/or the T-cell receptor diversity. In some embodiments, the diversity is in the immunoglobulin 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).

[00514] After dissection or digestion of tumor fragments, for example such as described in Step A of Figure 1, the resulting cells are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 3 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 7 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of 10 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this

primary cell population is cultured for a period of about 11 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells.

[00515] In a preferred embodiment, expansion of TILs may be performed using an initial bulk TIL expansion step (for example such as those described in Step B of Figure 1, which can include processes referred to as pre-REP) as described below and herein, followed by a second expansion (Step D, including processes referred to as rapid expansion protocol (REP) steps) as described below under Step D and herein, followed by optional cryopreservation, and followed by a second Step D (including processes referred to as restimulation REP steps) as described below and herein. The TILs obtained from this process may be optionally characterized for phenotypic characteristics and metabolic parameters as described herein.

[00516] 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.

[00517] 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) (Fig. 1), 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.

[00518] After preparation of the tumor fragments, the resulting cells (*i.e.*, fragments) are cultured in serum containing IL-2 under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the tumor digests are incubated in 2 mL wells in media comprising inactivated human AB serum (or, in some cases, as outlined herein, in the presence of aAPC cell population) with 6000 IU/mL of IL-2. This primary cell population is cultured for a period of days, generally from 10 to 14 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, the growth media

during the first expansion comprises IL-2 or a variant thereof. In some embodiments, the IL is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of 20-30×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 20×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 25×10^6 IU/mg for a 1 mg vial. In some embodiments the IL-2 stock solution has a specific activity of 30×10^6 IU/mg for a 1 mg vial. In some embodiments, the IL- 2 stock solution has a final concentration of 4-8×10^6 IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of 5-7×10^6 IU/mg of IL-2. In some embodiments, the IL- 2 stock solution has a final concentration of 6×10^6 IU/mg of IL-2. In some embodiments, the IL-2 stock solution is prepare as described in Example 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 an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium further comprises IL-2. In a preferred embodiment, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or about 8000 IU/mL of IL-2.

[00519] In some embodiments, first expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the first expansion

culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the first expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the cell culture medium further comprises IL-15. In a preferred embodiment, the cell culture medium comprises about 180 IU/mL of IL-15.

[00520] In some embodiments, first expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the first expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the cell culture medium comprises about 1 IU/mL of IL-21.

[00521] In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 μg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and

50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

TABLE 3: 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 NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:2 Muromonab light chain	QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC	60 120 180 213

[00522] 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.

[00523] 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.

[00524] 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) (Fig. 1), 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.

[00525] In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1, which can include those sometimes referred to as the pre-REP) process is shortened to 3-14 days, as discussed in the examples and figures. In some embodiments, the first expansion (including processes such as for example those described in Step B of Figure 1, which can include those sometimes referred to as the pre-REP) is shortened to 7 to 14 days, as discussed in the Examples and shown in Figures 4 and 5, as well as including for example, an expansion as described in Step B of Figure 1. In some embodiments, the first expansion of Step B is shortened to 10-14 days. In some embodiments, the first expansion is shortened to 11 days, as discussed in, for example, an expansion as described in Step B of Figure 1.

[00526] 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.

[00527] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the first expansion, including for example during a Step B processes according to Figure 1, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the first expansion. In some embodiments, IL-2, IL-15, and IL-21 as well as any combinations thereof can be included during Step B processes according to Figure 1 and as described herein.

[00528] In some embodiments, the first expansion (including processes referred to as the pre-REP; for example, Step B according to Figure 1) process is shortened to 3 to 14 days, as discussed in the examples and figures. In some embodiments, the first expansion of Step B is shortened to 7 to 14 days. In some embodiments, the first expansion of Step B is shortened to 10 to 14 days. In some embodiments, the first expansion is shortened to 11 days.

[00529] In some embodiments, the first expansion, for example, Step B according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

C. STEP C: First Expansion to Second Expansion Transition

[00530] In some cases, the bulk TIL population obtained from the first expansion, including for example the TIL population obtained from for example, Step B as indicated in Figure 1,

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

[00531] In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the first expansion (for example, from Step B as indicated in Figure 1) are not stored and proceed directly to the second expansion. In some embodiments, the TILs obtained from the first expansion are not cryopreserved after the first expansion and prior to the second expansion. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 3 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 4 days to 10 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 7 days to 14 days from when fragmentation occurs. In some embodiments, the transition from the first expansion to the second expansion occurs at about 14 days from when fragmentation occurs.

[00532] 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.

[00533] In some embodiments, the TILs are not stored after the first expansion and prior to the second expansion, and the TILs proceed directly to the second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 1). In some embodiments, the transition occurs in closed system, as described herein. In some embodiments, the TILs from the first expansion, the second population of TILs, proceeds directly into the second expansion with no transition period.

[00534] In some embodiments, the transition from the first expansion to the second expansion, for example, Step C according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single bioreactor is employed. In some embodiments, the single bioreactor employed is for example a G-REX -10 or a G-REX -100. In some embodiments, the closed system bioreactor is a single bioreactor.

1. Cytokines

[00535] 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.

[00536] Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILS is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in International Publication No. WO 2015/189356 and W International Publication No. WO 2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21 and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

TABLE 4: 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 EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN RWITFCQSII STLT	60 120 134
SEQ ID NO:4 Aldesleukin	PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW ITFSQSIIST LT	60 120 132
SEQ ID NO:5	MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI	60 120

recombinant human IL-4 (rhIL-4)	MREKYSKCSS	130
SEQ ID NO:6 recombinant human IL-7 (rhIL-7)	MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH	60 120 153
SEQ ID NO:7 recombinant human IL-15 (rhIL-15)	MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS	60 115
SEQ ID NO:8 recombinant human IL-21 (rhIL-21)	MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ HLSSRTHGSE DS	60 120 132

D. STEP D: Second Expansion

[00537] In some embodiments, the TIL cell population is expanded in number after harvest and initial bulk processing for example, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 1). This further expansion is referred to herein as the second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (REP; as well as processes as indicated in Step D of Figure 1). The second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container.

[00538] In some embodiments, the second expansion or second TIL expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 1) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days. In some embodiments, the second TIL expansion can proceed for about 7 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 8 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 9 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 10 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 11 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 12 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 13 days to about 14 days. In some embodiments, the second TIL expansion can proceed for about 14 days.

[00539] In an embodiment, the second expansion can be performed in a gas permeable container using the methods of the present disclosure (including for example, expansions referred to as REP; as well as processes as indicated in Step D of Figure 1). For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San Diego, CA, USA). TILs can be expanded to induce further stimulation of the TILs *in vitro* by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 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.

[00540] In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or between 8000 IU/mL of IL-2.

[00541] In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 μg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In some embodiments, the cell culture medium does not comprise OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

[00542] 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.

[00543] 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.

[00544] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the second expansion, including for example during a Step D processes according to Figure 1, as well as described herein. In some embodiments, a combination of IL-2, IL-15, and IL-21 are employed as a combination during the second expansion. In some embodiments, IL-2, IL-15,

and IL-21 as well as any combinations thereof can be included during Step D processes according to Figure 1 and as described herein.

[00545] 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).

[00546] In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15, about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the cell culture medium further comprises IL-15. In a preferred embodiment, the cell culture medium comprises about 180 IU/mL of IL-15.

[00547] In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media

comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the cell culture medium comprises about 1 IU/mL of IL-21.

[00548] In some embodiments the antigen-presenting feeder cells (APCs) are PBMCs. In an embodiment, the ratio of TILs to PBMCs and/or antigen-presenting cells in the rapid expansion and/or the second expansion is about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 100 and 1 to 200.

[00549] In an embodiment, REP and/or the second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. Media replacement is done (generally 2/3 media replacement via respiration with fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

[00550] 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.

[00551] In an embodiment, REP and/or the second expansion may be performed using T-175 flasks and gas permeable bags as previously described (Tran, *et al.*, *J. Immunother.* **2008,** *31,* 742-51; Dudley, *et al.*, *J. Immunother.* **2003,** *26,* 332-42) or gas permeable cultureware (G-Rex flasks). In some embodiments, the second expansion (including expansions referred to as rapid expansions) is performed in T-175 flasks, and about 1 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.

[00552] In an embodiment, the second expansion (which can include expansions referred to as REP, as well as those referred to in Step D of Figure 1) may be performed in 500 mL capacity gas permeable flasks with 100 cm gas-permeable silicon bottoms (G-Rex 100, commercially available from Wilson Wolf Manufacturing Corporation, New Brighton, MN, USA), 5 × 10^6 or 10 × 10^6 TIL may be cultured with PBMCs in 400 mL of 50/50 medium, supplemented with 5% human AB serum, 3000 IU per mL of IL-2 and 30 ng per ml of anti-CD3 (OKT3). The G-Rex 100 flasks may be incubated at 37°C in 5% 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.

[00553] In an embodiment, the second expansion (including expansions referred to as REP) is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, 30 mg/mL OKT3 anti-CD3 antibody and 3000 IU/mL IL-2 in 150 ml media. In some embodiments, media replacement is done until the cells are transferred to an alternative growth chamber. In some embodiments, 2/3 of the media is replaced by respiration with fresh media. In some embodiments, alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

[00554] In an embodiment, the second expansion (including expansions referred to as REP) is performed and further comprises a step wherein TILs are selected for superior tumor reactivity. Any selection method known in the art may be used. For example, the methods described in U.S. Patent Application Publication No. 2016/0010058 A1, the disclosures of

which are incorporated herein by reference, may be used for selection of TILs for superior tumor reactivity.

[00555] 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.

[00556] In some embodiments, the second expansion (including expansions referred to as REP) of TIL can be performed using T-175 flasks and gas-permeable bags as previously described (Tran KQ, Zhou J, Durflinger KH, et al., **2008**, *J Immunother.*, 31:742–751, and Dudley ME, Wunderlich JR, Shelton TE, et al. 2003, *J Immunother.*, 26:332–342) or gas-permeable G-Rex flasks. In some embodiments, the second expansion is performed using flasks. In some embodiments, the second expansion is performed using gas-permeable G-Rex flasks. In some embodiments, the second expansion is performed in T-175 flasks, and about 1 x 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 x 10^6 cells/mL.

[00557] 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) (Fig. 1), about 5x10^6 or 10x10^6 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 (491g) for 10 minutes. The TIL pellets can then be resuspended with 150 mL of fresh 50/50 medium with 3000 IU/ mL of IL-2 and added back to the original G-Rex 100 flasks. In embodiments where TILs are expanded serially in G-Rex 100 flasks, on day 7 the TIL in each G-Rex 100 are suspended in the 300 mL of media present in each flask and the cell suspension was divided into three 100 mL aliquots that are used to seed 3 G-Rex 100 flasks. Then 150 mL of AIM-V with 5% human AB serum and 3000 IU/mL of IL-2 is added to each flask. The G-Rex 100 flasks are incubated at 37°C in 5% 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.

[00558] 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).

[00559] 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.

[00560] In some embodiments, the second expansion, for example, Step D according to Figure 1, is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a single

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

1. Feeder Cells and Antigen Presenting Cells

[00561] In an embodiment, the second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 1, as well as those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

[00562] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

[00563] 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).

[00564] 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.

[00565] 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.

[00566] In some embodiments, the antigen-presenting feeder cells are PBMCs. In some embodiments, the antigen-presenting feeder cells are artificial antigen-presenting feeder cells. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

[00567] In an embodiment, the second expansion procedures described herein require a ratio of about $2.5x10^9$ feeder cells to about $100x10^6$ TILs. In another embodiment, the second expansion procedures described herein require a ratio of about $2.5x10^9$ feeder cells to about $50x10^6$ TILs. In yet another embodiment, the second expansion procedures described herein require about $2.5x10^9$ feeder cells to about $25x10^6$ TILs.

[00568] In an embodiment, the second expansion procedures described herein require an excess of feeder cells during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In an embodiment, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

[00569] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

[00570] In an embodiment, artificial antigen presenting cells are used in the second expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

[00571] 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.

[00572] Alternatively, using combinations of cytokines for the rapid expansion and or second expansion of TILS is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in International Publication No. WO 2015/189356 and W International Publication No. WO 2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21 and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

E. STEP E: Harvest TILS

[00573] After the second expansion step, cells can be harvested. In some embodiments the TILs are harvested after one, two, three, four or more expansion steps, for example as provided in Figure 1. In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 1.

[00574] TILs can be harvested in any appropriate and sterile manner, including for example by centrifugation. Methods for TIL harvesting are well known in the art and any such know methods can be employed with the present process. In some embodiments, TILS are harvest using an automated system.

[00575] 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.

[00576] In some embodiments, the harvest, for example, Step E according to Figure 1, is performed from a closed system bioreactor. In some embodiments, a closed system is

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

[00577] In some embodiments, Step E according to Figure 1, is performed according to the processes described in Example G. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in Example G is employed.

[00578] In some embodiments, TILs are harvested according to the methods described in Example G. In some embodiments, TILs between days 1 and 11 are harvested using the methods as described in Section 8.5 (referred to as the Day 11 TIL harvest in Example G). In some embodiments, TILs between days 12 and 22 are harvested using the methods as described in Section 8.12 (referred to as the Day 22 TIL harvest in Example G).

F. STEP F: Final Formulation/ Transfer to Infusion Bag

[00579] After Steps A through E as provided in an exemplary order in Figure 1 and as outlined in detailed above and herein are complete, cells are transferred to a container for use in administration to a patient. In some embodiments, once a therapeutically sufficient number of TILs are obtained using the expansion methods described above, they are transferred to a container for use in administration to a patient.

[00580] In an embodiment, TILs expanded using APCs of the present disclosure are administered to a patient as a pharmaceutical composition. In an embodiment, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded using PBMCs of the present disclosure may be administered by any suitable route as known in the art. In some embodiments, the T-cells are administered as a single intra-arterial or intravenous infusion, which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic.

G. Optional Cell Medium Components

1. Anti-CD3 Antibodies

[00581] In some embodiments, the culture media used in expansion methods described herein (including those referred to as REP, see for example, Figure 1) also includes an anti-CD3 antibody. An anti-CD3 antibody in combination with IL-2 induces T cell activation and

cell division in the TIL population. This effect can be seen with full length antibodies as well as Fab and F(ab')2 fragments, with the former being generally preferred; see, *e.g.,* Tsoukas *et al.*, *J. Immunol.* **1985,** *135,* 1719, hereby incorporated by reference in its entirety.

[00582] As will be appreciated by those in the art, there are a number of suitable anti-human CD3 antibodies that find use in the invention, including anti-human CD3 polyclonal and monoclonal antibodies from various mammals, including, but not limited to, murine, human, primate, rat, and canine antibodies. In particular embodiments, the OKT3 anti-CD3 antibody is used (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA).

TABLE 5: 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 NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:2 Muromonab light chain	QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC	60 120 180 213

2. 4-1BB (CD137) AGONISTS

[00583] In an embodiment, the TNFRSF agonist is a 4-1BB (CD137) agonist. The 4-1BB agonist may be any 4-1BB binding molecule known in the art. The 4-1BB binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian 4-1BB. The 4-1BB agonists or 4-1BB binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The 4-1BB agonist or 4-1BB binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to 4-1BB. In an embodiment, the 4-1BB agonist is

an antigen binding protein that is a fully human antibody. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a humanized antibody. In some embodiments, 4-1BB agonists for use in the presently disclosed methods and compositions include anti-4-1BB antibodies, human anti-4-1BB antibodies, mouse anti-4-1BB antibodies, mammalian anti-4-1BB antibodies, monoclonal anti-4-1BB antibodies, polyclonal anti-4-1BB antibodies, chimeric anti-4-1BB antibodies, anti-4-1BB adnectins, anti-4-1BB domain antibodies, single chain anti-4-1BB fragments, heavy chain anti-4-1BB fragments, light chain anti-4-1BB fragments, anti-4-1BB fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. Agonistic anti-4-1BB antibodies are known to induce strong immune responses. Lee, *et al.*, *PLOS One* **2013**, *8,* e69677. In a preferred embodiment, the 4-1BB agonist is an agonistic, anti-4-1BB humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line). In an embodiment, the 4-1BB agonist is EU-101 (Eutilex Co. Ltd.), utomilumab, or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof. In a preferred embodiment, the 4-1BB agonist is utomilumab or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof.

[00584] In a preferred embodiment, the 4-1BB agonist or 4-1BB binding molecule may also be a fusion protein. In a preferred embodiment, a multimeric 4-1BB agonist, such as a trimeric or hexameric 4-1BB agonist (with three or six ligand binding domains), may induce superior receptor (4-1BBL) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

[00585] Agonistic 4-1BB antibodies and fusion proteins are known to induce strong immune responses. In a preferred embodiment, the 4-1BB agonist is a monoclonal antibody or fusion protein that binds specifically to 4-1BB antigen in a manner sufficient to reduce toxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the

4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein which abrogates Fc region functionality.

[00586] In some embodiments, the 4-1BB agonists are characterized by binding to human 4-1BB (SEQ ID NO:9) with high affinity and agonistic activity. In an embodiment, the 4-1BB agonist is a binding molecule that binds to human 4-1BB (SEQ ID NO:9). In an embodiment, the 4-1BB agonist is a binding molecule that binds to murine 4-1BB (SEQ ID NO:10). The amino acid sequences of 4-1BB antigen to which a 4-1BB agonist or binding molecule binds are summarized in Table 6.

TABLE 6. Amino acid sequences of 4-1BB antigens.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:9 human 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Homo sapiens)	MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE PGHSPQIISF FLALTSTALL FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG CSCRFPEEEE GGCEL	60 120 180 240 255
SEQ ID NO:10 murine 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Mus musculus)	MGNNCYNVVV IVLLLVGCEK VGAVQNSCDN CQPGTFCRKY NPVCKSCPPS TFSSIGGQPN CNICRVCAGY FRFKKFCSST HNAECECIEG FHCLGPQCTR CEKDCRPGQE LTKQGCKTCS LGTFNDQNGT GVCRPWTNCS LDGRSVLKTG TTEKDVVCGP PVVSFSPSTT ISVTPEGGPG GHSLQVLTLF LALTSALLLA LIFITLLFSV LKWIRKKFPH IFKQPFKKTT GAAQEEDACS CRCPQEEEGG GGGYEL	60 120 180 240 256

[00587] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds human or murine 4-1BB with a K_D of about 100 pM or lower, binds human or murine 4-1BB with a K_D of about 90 pM or lower, binds human or murine 4-1BB with a K_D of about 80 pM or lower, binds human or murine 4-1BB with a K_D of about 70 pM or lower, binds human or murine 4-1BB with a K_D of about 60 pM or lower, binds human or murine 4-1BB with a K_D of about 50 pM or lower, binds human or murine 4-1BB with a K_D of about 40 pM or lower, or binds human or murine 4-1BB with a K_D of about 30 pM or lower.

[00588] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human or murine 4-1BB with a k_{assoc} of about 1×10^6 1/M·s or faster.

[00589] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a k_{dissoc} of about 2×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower , binds to human or murine 4-1BB with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human or murine 4-1BB with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human or murine 4-1BB with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[00590] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with an IC_{50} of about 10 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 9 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 8 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 7 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 6 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 5 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 4 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 3 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 2 nM or lower, or binds to human or murine 4-1BB with an IC_{50} of about 1 nM or lower.

[00591] In a preferred embodiment, the 4-1BB agonist is utomilumab, also known as PF-05082566 or MOR-7480, or a fragment, derivative, variant, or biosimilar thereof. Utomilumab is available from Pfizer, Inc. Utomilumab is an immunoglobulin G2-lambda, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor (TNFR) superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of utomilumab are set forth in Table EE. Utomilumab comprises glycosylation sites at Asn59 and Asn292; heavy chain intrachain disulfide bridges at positions 22-96 (V_H-V_L), 143-199 (C_H1-C_L), 256-316 (C_H2) and 362-420 (C_H3); light chain intrachain disulfide bridges at positions 22'-87' (V_H-V_L) and 136'-195' (C_H1-C_L); interchain heavy chain-heavy chain disulfide bridges at IgG2A isoform positions 218-218, 219-219, 222-222, and 225-225, at IgG2A/B isoform positions 218-130, 219-219, 222-222, and 225-225, and at IgG2B isoform positions 219-130 (2), 222-222, and 225-225; and interchain heavy chain-light chain disulfide bridges at IgG2A isoform positions 130-213' (2), IgG2A/B

isoform positions 218-213' and 130-213', and at IgG2B isoform positions 218-213' (2). The preparation and properties of utomilumab and its variants and fragments are described in U.S. Patent Nos. 8,821,867; 8,337,850; and 9,468,678, and International Patent Application Publication No. WO 2012/032433 A1, the disclosures of each of which are incorporated by reference herein. Preclinical characteristics of utomilumab are described in Fisher, *et al.*, *Cancer Immunolog. & Immunother.* **2012,** *61,* 1721-33. Current clinical trials of utomilumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02444793, NCT01307267, NCT02315066, and NCT02554812.

[00592] In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:11 and a light chain given by SEQ ID NO:12. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively.

[00593] In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of utomilumab. In an embodiment, the 4-1BB agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:13, and the 4-1BB agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:14, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L

regions that are each at least 97% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14.

[00594] In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:18, SEQ ID NO:19, and SEQ ID NO:20, respectively, and conservative amino acid substitutions thereof.

[00595] In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to utomilumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the biosimilar is provided as a composition which further

comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab.

TABLE 7. Amino acid sequences for 4-1BB agonist antibodies related to utomilumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:11 heavy chain for utomilumab	EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMGK IYPGDSYTNY SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCARGY GIFDYWGQGT LVTVSSASTK GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVHTFP AVLQSSGLYS LSSVVTVPSS NFGTQTYTCN VDHKPSNTKV DKTVERKCCV ECPPCPAPPV AGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTFRVVSV LTVVHQDWLN GKEYKCKVSN KGLPAPIEKT ISKTKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PMLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP G	60 120 180 240 300 360 420 441
SEQ ID NO:12 light chain for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVLGQ PKAAPSVTLF PPSSEELQAN KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTPSKQS NNKYAASSYL SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS	60 120 180 214
SEQ ID NO:13 heavy chain variable region for utomilumab	EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMG KIYPGDSYTN YSPSFQGQVT ISADKSISTA YLQWSSLKAS DTAMYYCARG YGIFDYWGQ GTLVTVSS	60 118
SEQ ID NO:14 light chain variable region for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVL	60 108
SEQ ID NO:15 heavy chain CDR1 for utomilumab	STYWIS	6
SEQ ID NO:16 heavy chain CDR2 for utomilumab	KIYPGDSYTN YSPSFQG	17
SEQ ID NO:17 heavy chain CDR3 for utomilumab	RGYGIFDY	8
SEQ ID NO:18 light chain CDR1 for utomilumab	SGDNIGDQYA H	11
SEQ ID NO:19 light chain CDR2 for utomilumab	QDKNRPS	7
SEQ ID NO:20 light chain CDR3 for utomilumab	ATYTGFGSLA V	11

[00596] In a preferred embodiment, the 4-1BB agonist is the monoclonal antibody urelumab, also known as BMS-663513 and 20H4.9.h4a, or a fragment, derivative, variant, or biosimilar thereof. Urelumab is available from Bristol-Myers Squibb, Inc., and Creative Biolabs, Inc. Urelumab is an immunoglobulin G4-kappa, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of urelumab are set forth in Table EE. Urelumab comprises N-glycosylation sites at positions 298 (and 298'); heavy chain intrachain disulfide bridges at positions 22-95 (V_H-V_L), 148-204 (C_H1-C_L), 262-322 (C_H2) and 368-426 (C_H3) (and at positions 22''-95'', 148''-204'', 262''-322'', and 368''-426''); light chain intrachain disulfide bridges at positions 23'-88' (V_H-V_L) and 136'-196'

(C_H1-C_L) (and at positions 23'''-88''' and 136'''-196'''); interchain heavy chain-heavy chain disulfide bridges at positions 227-227'' and 230-230''; and interchain heavy chain-light chain disulfide bridges at 135-216' and 135''-216'''. The preparation and properties of urelumab and its variants and fragments are described in U.S. Patent Nos. 7,288,638 and 8,962,804, the disclosures of which are incorporated by reference herein. The preclinical and clinical characteristics of urelumab are described in Segal, *et al.*, *Clin. Cancer Res.* **2016,** *available at* http:/dx.doi.org/ 10.1158/1078-0432.CCR-16-1272. Current clinical trials of urelumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT01775631, NCT02110082, NCT02253992, and NCT01471210.

[00597] In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:21 and a light chain given by SEQ ID NO:22. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively.

[00598] In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of urelumab. In an embodiment, the 4-1BB agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:23, and the 4-1BB agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:24, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:23

and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24.

[00599] In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:25, SEQ ID NO:26, and SEQ ID NO:27, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30, respectively, and conservative amino acid substitutions thereof.

[00600] In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to urelumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab.

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

TABLE 8: Amino acid sequences for 4-1BB agonist antibodies related to urelumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:21 heavy chain for urelumab	QVQLQQWGAG LLKPSETLSL TCAVYGGSFS GYYWSWIRQS PEKGLEWIGE INHGGYVTYN PSLESRVTIS VDTSKNQFSL KLSSVTAADT AVYYCARDYG PGNYDWYFDL WGRGTLVTVS SASTKGPSVF PLAPCSRSTS ESTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTK TYTCNVDHKP SNTKVDKRVE SKYGPPCPPC PAPEFLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK	60 120 180 240 300 360 420 448
SEQ ID NO:22 light chain for urelumab	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPALTF CGGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC	60 120 180 216
SEQ ID NO:23 variable heavy chain for urelumab	MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YYWSWIRQSP EKGLEWIGEI NHGGYVTYNP SLESRVTISV DTSKNQFSLK LSSVTAADTA VYYCARDYGP	60 120
SEQ ID NO:24 variable light chain for urelumab	MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ	60 110
SEQ ID NO:25 heavy chain CDR1 for urelumab	GYYWS	5
SEQ ID NO:26 heavy chain CDR2 for urelumab	EINHGGYVTY NPSLES	16
SEQ ID NO:27 heavy chain CDR3 for urelumab	DYGPGNYDWY FDL	13
SEQ ID NO:28 light chain CDR1 for urelumab	RASQSVSSYL A	11
SEQ ID NO:29 light chain CDR2 for urelumab	DASNRAT	7
SEQ ID NO:30 light chain CDR3 for urelumab	QQRSDWPPAL T	11

[00601] In an embodiment, the 4-1BB agonist is selected from the group consisting of 1D8, 3Elor, 4B4 (BioLegend 309809), H4-1BB-M127 (BD Pharmingen 552532), BBK2 (Thermo Fisher MS621PABX), 145501 (Leinco Technologies B591), the antibody produced by cell line deposited as ATCC No. HB-11248 and disclosed in U.S. Patent No. 6,974,863, 5F4 (BioLegend 31 1503), C65-485 (BD Pharmingen 559446), antibodies disclosed in U.S. Patent Application Publication No. US 2005/0095244, antibodies disclosed in U.S. Patent No. 7,288,638 (such as 20H4.9-IgGl (BMS-663031)), antibodies disclosed in U.S. Patent No. 6,887,673 (such as 4E9 or BMS-554271), antibodies disclosed in U.S. Patent No. 7,214,493, antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in U.S. Patent No. 6,905,685 (such as 4E9 or BMS-554271),

antibodies disclosed in U.S. Patent No. 6,362,325 (such as 1D8 or BMS-469492; 3H3 or BMS-469497; or 3El), antibodies disclosed in U.S. Patent No. 6,974,863 (such as 53A2); antibodies disclosed in U.S. Patent No. 6,210,669 (such as 1D8, 3B8, or 3El), antibodies described in U.S. Patent No. 5,928,893, antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in International Patent Application Publication Nos. WO 2012/177788, WO 2015/119923, and WO 2010/042433, and fragments, derivatives, conjugates, variants, or biosimilars thereof, wherein the disclosure of each of the foregoing patents or patent application publications is incorporated by reference here.

[00602] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein described in International Patent Application Publication Nos. WO 2008/025516 A1, WO 2009/007120 A1, WO 2010/003766 A1, WO 2010/010051 A1, and WO 2010/078966 A1; U.S. Patent Application Publication Nos. US 2011/0027218 A1, US 2015/0126709 A1, US 2011/0111494 A1, US 2015/0110734 A1, and US 2015/0126710 A1; and U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

[00603] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof (see, Figure 50). In structures I-A and I-B, the cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from *e.g.*, 4-1BBL or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second triavelent protein through IgG1-Fc (including C_H3 and C_H2 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. Any scFv domain design may be used, such as those described in de Marco, *Microbial Cell Factories*, **2011,** *10*, 44; Ahmad, *et al.*, *Clin. & Dev. Immunol.* **2012,** 980250; Monnier, *et al.*, *Antibodies,* **2013,** *2,* 193-208; or in references incorporated elsewhere herein. Fusion protein structures of this

form are described in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

[00604] Amino acid sequences for the other polypeptide domains of structure I-A are given in Table GG. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides.

TABLE 9: Amino acid sequences for TNFRSF fusion proteins, including 4-1BB fusion proteins, with C-terminal Fc-antibody fragment fusion protein design (structure I-A).

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:31 Fc domain	KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 230
SEQ ID NO:32 linker	GGPGSSKSCD KTHTCPPCPA PE	22
SEQ ID NO:33 linker	GGSGSSKSCD KTHTCPPCPA PE	22
SEQ ID NO:34 linker	GGPGSSSSSS SKSCDKTHTC PPCPAPE	27
SEQ ID NO:35 linker	GGSGSSSSSS SKSCDKTHTC PPCPAPE	27
SEQ ID NO:36 linker	GGPGSSSSSS SSSKSCDKTH TCPPCPAPE	29
SEQ ID NO:37 linker	GGSGSSSSSS SSSKSCDKTH TCPPCPAPE	29
SEQ ID NO:38 linker	GGPGSSGSGS SDKTHTCPPC PAPE	24
SEQ ID NO:39 linker	GGPGSSGSGS DKTHTCPPCP APE	23
SEQ ID NO:40 linker	GGPSSSGSDK THTCPPCPAP E	21
SEQ ID NO:41 linker	GGSSSSSSSS GSDKTHTCPP CPAPE	25

[00605] Amino acid sequences for the other polypeptide domains of structure I-B are given in Table HH. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

TABLE 10: Amino acid sequences for TNFRSF fusion proteins, including 4-1BB fusion proteins, with N-terminal Fc-antibody fragment fusion protein design (structure I-B).

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:42 Fc domain	METDTLLLWV LLLWVPAGNG DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE	60 120 180

	WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG	240 246
SEQ ID NO:43 linker	SGSGSGSGSG S	11
SEQ ID NO:44 linker	SSSSSSGSGS GS	12
SEQ ID NO:45 linker	SSSSSSGSGS GSGSGS	16

[00606] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains selected from the group consisting of a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain of urelumab, a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table GG, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[00607] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:46. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a soluble 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:47.

[00608] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the V_H and V_L sequences given in Table 11, wherein the V_H and V_L domains are connected by a linker.

TABLE 11: Additional polypeptide domains useful as 4-1BB binding domains in fusion proteins or as scFv 4-1BB agonist antibodies.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:46 4-1BBL	MEYASDASLD PEAPWPPAPR ARACRVLPWA LVAGLLLLLL LAAACAVFLA CPWAVSGARA SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAALA LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ GATVLGLFRV TPEIPAGLPS PRSE	60 120 180 240 254
SEQ ID NO:47 4-1BBL soluble domain	LRQGMFAQLV AQNVLLIDGP LSWYSDPGLA GVSLTGGLSY KEDTKELVVA KAGVYYVFFQ LELRRVVAGE GSGSVSLALH LQPLRSAAGA AALALTVDLP PASSEARNSA FGFQGRLLHL SAGQRLGVHL HTEARARHAW QLTQGATVLG LFRVTPEIPA GLPSPRSE	60 120 168
SEQ ID NO:48 variable heavy chain for 4B4-1-1 version 1	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVS	60 118
SEQ ID NO:49 variable light chain for 4B4-1-1 version 1	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIK	60 107
SEQ ID NO:50 variable heavy chain for 4B4-1-1 version 2	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVSA	60 119
SEQ ID NO:51 variable light chain for 4B4-1-1 version 2	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIKR	60 108
SEQ ID NO:52 variable heavy chain for H39E3-2	MDWTWRILFL VAAATGAHSE VQLVESGGGL VQPGGSLRLS CAASGFTFSD YWMSWVRQAP GKGLEWVADI KNDGSYTNYA PSLTNRFTIS RDNAKNSLYL QMNSLRAEDT AVYYCARELT	60 120
SEQ ID NO:53 variable light chain for H39E3-2	MEAPAQLLFL LLLWLPDTTG DIVMTQSPDS LAVSLGERAT INCKSSQSLL SSGNQKNYL WYQQKPGQPP KLLIYYASTR QSGVPDRFSG SGSGTDFTLT ISSLQAEDVA	60 110

[00609] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain, wherein each of the soluble 4-1BB domains lacks a stalk region (which contributes to trimerisation and provides a certain distance to the cell membrane, but is not part of the 4-1BB binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

[00610] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain,

(iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein each TNF superfamily cytokine domain is a 4-1BB binding domain.

[00611] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic scFv antibody comprising any of the foregoing V_H domains linked to any of the foregoing V_L domains.

[00612] In an embodiment, the 4-1BB agonist is BPS Bioscience 4-1BB agonist antibody catalog no. 79097-2, commercially available from BPS Bioscience, San Diego, CA, USA. In an embodiment, the 4-1BB agonist is Creative Biolabs 4-1BB agonist antibody catalog no. MOM-18179, commercially available from Creative Biolabs, Shirley, NY, USA.

3. OX40 (CD134) AGONISTS

[00613] In an embodiment, the TNFRSF agonist is an OX40 (CD134) agonist. The OX40 agonist may be any OX40 binding molecule known in the art. The OX40 binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian OX40. The OX40 agonists or OX40 binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The OX40 agonist or OX40 binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to OX40. In an embodiment, the OX40 agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the OX40 agonist is an antigen binding protein that is a humanized antibody. In some embodiments, OX40 agonists for use in the presently disclosed methods and compositions include anti-OX40 antibodies, human anti-OX40 antibodies, mouse anti-OX40 antibodies, mammalian anti-OX40 antibodies, monoclonal anti-OX40 antibodies, polyclonal anti-OX40 antibodies, chimeric anti-OX40 antibodies, anti-OX40 adnectins, anti-OX40 domain antibodies, single chain anti-OX40 fragments, heavy chain anti-OX40 fragments, light chain anti-OX40 fragments, anti-OX40 fusion proteins, and fragments, derivatives, conjugates, variants, or

biosimilars thereof. In a preferred embodiment, the OX40 agonist is an agonistic, anti-OX40 humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line).

[00614] In a preferred embodiment, the OX40 agonist or OX40 binding molecule may also be a fusion protein. OX40 fusion proteins comprising an Fc domain fused to OX40L are described, for example, in Sadun, *et al.*, *J. Immunother.* **2009,** *182,* 1481-89. In a preferred embodiment, a multimeric OX40 agonist, such as a trimeric or hexameric OX40 agonist (with three or six ligand binding domains), may induce superior receptor (OX40L) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

[00615] Agonistic OX40 antibodies and fusion proteins are known to induce strong immune responses. Curti, *et al.*, *Cancer Res.* **2013**, *73,* 7189-98. In a preferred embodiment, the OX40 agonist is a monoclonal antibody or fusion protein that binds specifically to OX40 antigen in a manner sufficient to reduce toxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein which abrogates Fc region functionality.

[00616] In some embodiments, the OX40 agonists are characterized by binding to human OX40 (SEQ ID NO:54) with high affinity and agonistic activity. In an embodiment, the OX40 agonist is a binding molecule that binds to human OX40 (SEQ ID NO:54). In an embodiment, the OX40 agonist is a binding molecule that binds to murine OX40 (SEQ ID NO:55). The amino acid sequences of OX40 antigen to which an OX40 agonist or binding molecule binds are summarized in Table 12.

TABLE 12: Amino acid sequences of OX40 antigens.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:54 human OX40 (Homo sapiens)	MCVGARRLGR GPCAALLLLG LGLSTVTGLH CVGDTYPSND RCCHECRPGN GMVSRCSRSQ NTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSERKQLCT ATQDTVCRCR AGTQPLDSYK PGVDCAPCPP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQ GPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI	60 120 180 240 277
SEQ ID NO:55 murine OX40 (Mus musculus)	MYVWVQQPTA LLLLGLTLGV TARRLNCVKH TYPSGHKCCR ECQPGHGMVS RCDHTRDTLC HPCETGFYNE AVNYDTCKQC TQCNHRSGSE LKQNCTPTQD TVCRCRPGTQ PRQDSGYKLG VDCVPCPPGH FSPGNNQACK PWTNCTLSGK QTRHPASDSL DAVCEDRSLL ATLLWETQRP TFRPTTVQST TVWPRTSELP SPPTLVTPEG PAFAVLLGLG LGLLAPLTVL LALYLLRKAW RLPNTPKPCW GNSFRTPIQE EHTDAHFTLA KI	60 120 180 240 272

[00617] In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds human or murine OX40 with a K_D of about 100 pM or lower, binds human or murine OX40 with a K_D of about 90 pM or lower, binds human or murine OX40 with a K_D of about 80 pM or lower, binds human or murine OX40 with a K_D of about 70 pM or lower, binds human or murine OX40 with a K_D of about 60 pM or lower, binds human or murine OX40 with a K_D of about 50 pM or lower, binds human or murine OX40 with a K_D of about 40 pM or lower, or binds human or murine OX40 with a K_D of about 30 pM or lower.

[00618] In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human or murine OX40 with a k_{assoc} of about 1×10^6 1/M·s or faster.

[00619] In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a k_{dissoc} of about 2×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower , binds to human or murine OX40 with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human or murine OX40 with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human or murine OX40 with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[00620] In some embodiments, the compositions, processes and methods described include OX40 agonist that binds to human or murine OX40 with an IC_{50} of about 10 nM or lower, binds to human or murine OX40 with an IC_{50} of about 9 nM or lower, binds to human or murine OX40 with an IC_{50} of about 8 nM or lower, binds to human or murine OX40 with an IC_{50} of about 7 nM or lower, binds to human or murine OX40 with an IC_{50} of about 6 nM or lower, binds to human or murine OX40 with an IC_{50} of about 5 nM or lower, binds to human or murine OX40 with an IC_{50} of about 4 nM or lower, binds to human or murine OX40 with an IC_{50} of about 3 nM or lower, binds to human or murine OX40 with an IC_{50} of about 2 nM or lower, or binds to human or murine OX40 with an IC_{50} of about 1 nM or lower.

[00621] In some embodiments, the OX40 agonist is tavolixizumab, also known as MEDI0562 or MEDI-0562. Tavolixizumab is available from the MedImmune subsidiary of AstraZeneca, Inc. Tavolixizumab is immunoglobulin G1-kappa, anti-[*Homo sapiens* TNFRSF4 (tumor necrosis factor receptor (TNFR) superfamily member 4, OX40, CD134)], humanized and chimeric monoclonal antibody. The amino acid sequences of tavolixizumab are set forth in Table KK. Tavolixizumab comprises N-glycosylation sites at positions 301 and 301'', with fucosylated complex bi-antennary CHO-type glycans; heavy chain intrachain disulfide bridges at positions 22-95 (V_H-V_L), 148-204 (C_H1-C_L), 265-325 (C_H2) and 371-429 (C_H3) (and at positions 22''-95'', 148''-204'', 265''-325'', and 371''-429''); light chain intrachain disulfide bridges at positions 23'-88' (V_H-V_L) and 134'-194' (C_H1-C_L) (and at positions 23'''-88''' and 134'''-194'''); interchain heavy chain-heavy chain disulfide bridges at positions 230-230'' and 233-233''; and interchain heavy chain-light chain disulfide bridges at 224-214' and 224''-214'''. Current clinical trials of tavolixizumab in a variety of solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02318394 and NCT02705482.

[00622] In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:56 and a light chain given by SEQ ID NO:57. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist

comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively.

[00623] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of tavolixizumab. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:58, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:59, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, an OX40 agonist comprises an scFv antibody comprising V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59.

[00624] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:63, SEQ ID NO:64, and SEQ ID NO:65, respectively, and conservative amino acid substitutions thereof.

[00625] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to tavolixizumab. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or

reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab.

TABLE 13: Amino acid sequences for OX40 agonist antibodies related to tavolixizumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:56 heavy chain for tavolixizumab	QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVTVS SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K	60 120 180 240 300 360 420 451
SEQ ID NO:57 light chain for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	60 120 180 214
SEQ ID NO:58 heavy chain variable region for tavolixizumab	QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVT	60 118
SEQ ID NO:59 light chain variable region for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKR	60 108
SEQ ID NO:60 heavy chain CDR1 for tavolixizumab	GSFSSGYWN	9

SEQ ID NO:61 heavy chain CDR2 for tavolixizumab	YIGYISYNGI TYH	13
SEQ ID NO:62 heavy chain CDR3 for tavolixizumab	RYKYDYDGGH AMDY	14
SEQ ID NO:63 light chain CDR1 for tavolixizumab	QDISNYLN	8
SEQ ID NO:64 light chain CDR2 for tavolixizumab	LLIYYTSKLH S	11
SEQ ID NO:65 light chain CDR3 for tavolixizumab	QQGSALPW	8

[00626] In some embodiments, the OX40 agonist is 11D4, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 11D4 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 11D4 are set forth in Table LL.

[00627] In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:66 and a light chain given by SEQ ID NO:67. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively.

[00628] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 11D4. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:68, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:69, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises

V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively.

[00629] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:72, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:73, SEQ ID NO:74, and SEQ ID NO:75, respectively, and conservative amino acid substitutions thereof.

[00630] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 11D4. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product,

wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4.

TABLE 14: Amino acid sequences for OX40 agonist antibodies related to 11D4.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:66 heavy chain for 11D4	EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSSAS TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSNFGTQTYT CNVDHKPSNT KVDKTVERKC CVECPPCPAP PVAGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTFRVV SVLTVVHQDW LNGKEYKCKV SNKGLPAPIE KTISKTKGQP REPQVYTLPP SREEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPMLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK	60 120 180 240 300 360 420 444
SEQ ID NO:67 light chain for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	60 120 180 214
SEQ ID NO:68 heavy chain variable region for 11D4	EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSS	60 118
SEQ ID NO:69 light chain variable region for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIK	60 107
SEQ ID NO:70 heavy chain CDR1 for 11D4	SYSMN	5
SEQ ID NO:71 heavy chain CDR2 for 11D4	YISSSSSTID YADSVKG	17
SEQ ID NO:72 heavy chain CDR3 for 11D4	ESGWYLFDY	9
SEQ ID NO:73 light chain CDR1 for 11D4	RASQGISSWL A	11
SEQ ID NO:74 light chain CDR2 for 11D4	AASSLQS	7
SEQ ID NO:75 light chain CDR3 for 11D4	QQYNSYPPT	9

[00631] In some embodiments, the OX40 agonist is 18D8, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 18D8 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 18D8 are set forth in Table MM.

[00632] In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:76 and a light chain given by SEQ ID NO:77. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively, or antigen binding fragments, Fab fragments, single-chain variable

fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively.

[00633] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 18D8. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:78, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:79, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively.

[00634] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:80, SEQ ID NO:81, and SEQ ID NO:82, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:83, SEQ ID NO:84, and SEQ ID NO:85, respectively, and conservative amino acid substitutions thereof.

[00635] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 18D8. In an embodiment,

the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8.

TABLE 15: Amino acid sequences for OX40 agonist antibodies related to 18D8.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:76 heavy chain for 18D8	EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV TVSSASTKGP SVFPLAPCSR STSESTAALG CLVKDYFPEP VTVSWNSGAL TSGVHTFPAV LQSSGLYSLS SVVTVPSSNF GTQTYTCNVD HKPSNTKVDK TVERKCCVEC PPCPAPPVAG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVQFNW YVDGVEVHNA KTKPREEQFN STFRVVSVLT VVHQDWLNGK EYKCKVSNKG LPAPIEKTIS KTKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPM LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:77 light chain for 18D8	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC	60 120 180 213
SEQ ID NO:78 heavy chain variable region for 18D8	EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV TVSS	60 120 124
SEQ ID NO:79 light chain variable region for 18D8	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIK	60 106

SEQ ID NO:80 heavy chain CDR1 for 18D8	DYAMH	5
SEQ ID NO:81 heavy chain CDR2 for 18D8	GISWNSGSIG YADSVKG	17
SEQ ID NO:82 heavy chain CDR3 for 18D8	DQSTADYYFY YGMDV	15
SEQ ID NO:83 light chain CDR1 for 18D8	RASQSVSSYL A	11
SEQ ID NO:84 light chain CDR2 for 18D8	DASNRAT	7
SEQ ID NO:85 light chain CDR3 for 18D8	QQRSNWPT	8

[00636] In some embodiments, the OX40 agonist is Hu119-122, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu119-122 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu119-122 are set forth in Table NN.

[00637] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu119-122. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:86, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:87, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively.

[00638] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:90, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:91, SEQ ID NO:92, and SEQ ID NO:93, respectively, and conservative amino acid substitutions thereof.

[00639] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu119-122. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122.

TABLE 16: Amino acid sequences for OX40 agonist antibodies related to Hu119-122.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:86 heavy chain variable region for Hu119-122	EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS	60 120
SEQ ID NO:87 light chain variable region for Hu119-122	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K	60 111
SEQ ID NO:88 heavy chain CDR1 for Hu119-122	SHDMS	5
SEQ ID NO:89 heavy chain CDR2 for Hu119-122	AINSDGGSTY YPDTMER	17

SEQ ID NO:90 heavy chain CDR3 for Hu119-122	HYDDYYAWFA Y	11
SEQ ID NO:91 light chain CDR1 for Hu119-122	RASKSVSTSG YSYMH	15
SEQ ID NO:92 light chain CDR2 for Hu119-122	LASNLES	7
SEQ ID NO:93 light chain CDR3 for Hu119-122	QHSRELPLT	9

[00640] In some embodiments, the OX40 agonist is Hu106-222, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu106-222 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu106-222 are set forth in Table OO.

[00641] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu106-222. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:94, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:95, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively.

[00642] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:99, SEQ ID NO:100, and SEQ ID NO:101, respectively, and conservative amino acid substitutions thereof.

[00643] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu106-222. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222.

TABLE 17: Amino acid sequences for OX40 agonist antibodies related to Hu106-222.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:94 heavy chain variable region for Hu106-222	QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV SS	60 120 122
SEQ ID NO:95 light chain variable region for Hu106-222	DIQMTQSPSS LSASVGDRVT ITCKASQDVS TAVAWYQQKP GKAPKLLIYS ASYLYTGVPS RFSGSGSGTD FTFTISSLQP EDIATYYCQQ HYSTPRTFGQ GTKLEIK	60 107
SEQ ID NO:96 heavy chain CDR1 for Hu106-222	DYSMH	5
SEQ ID NO:97 heavy chain CDR2 for Hu106-222	WINTETGEPT YADDFKG	17

SEQ ID NO:98 heavy chain CDR3 for Hu106-222	PYYDYVSYYA MDY	13
SEQ ID NO:99 light chain CDR1 for Hu106-222	KASQDVSTAV A	11
SEQ ID NO:100 light chain CDR2 for Hu106-222	SASYLYT	7
SEQ ID NO:101 light chain CDR3 for Hu106-222	QQHYSTPRT	9

[00644] In some embodiments, the OX40 agonist antibody is MEDI6469 (also referred to as 9B12). MEDI6469 is a murine monoclonal antibody. Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585. In some embodiments the OX40 agonist is an antibody produced by the 9B12 hybridoma, deposited with Biovest Inc. (Malvern, MA, USA), as described in Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585, the disclosure of which is hereby incorporated by reference in its entirety. In some embodiments, the antibody comprises the CDR sequences of MEDI6469. In some embodiments, the antibody comprises a heavy chain variable region sequence and/or a light chain variable region sequence of MEDI6469.

[00645] In an embodiment, the OX40 agonist is L106 BD (Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises the CDRs of antibody L106 (BD Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody L106 (BD Pharmingen Product #340420). In an embodiment, the OX40 agonist is ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises the CDRs of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In an embodiment, the OX40 agonist is the murine monoclonal antibody anti-mCD134/mOX40 (clone OX86), commercially available from InVivoMAb, BioXcell Inc, West Lebanon, NH.

[00646] In an embodiment, the OX40 agonist is selected from the OX40 agonists described in International Patent Application Publication Nos. WO 95/12673, WO 95/21925, WO 2006/121810, WO 2012/027328, WO 2013/028231, WO 2013/038191, and WO 2014/148895; European Patent Application EP 0672141; U.S. Patent Application Publication Nos. US 2010/136030, US 2014/377284, US 2015/190506, and US 2015/132288 (including clones 20E5 and 12H3); and U.S. Patent Nos. 7,504,101, 7,550,140, 7,622,444, 7,696,175,

7,960,515, 7,961,515, 8,133,983, 9,006,399, and 9,163,085, the disclosure of each of which is incorporated herein by reference in its entirety.

[00647] In an embodiment, the OX40 agonist is an OX40 agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof. The properties of structures I-A and I-B are described above and in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein. Amino acid sequences for the polypeptide domains of structure I-A are given in Table GG. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides. Likewise, amino acid sequences for the polypeptide domains of structure I-B are given in Table HH. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

[00648] In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains selected from the group consisting of a variable heavy chain and variable light chain of tavolixizumab, a variable heavy chain and variable light chain of 11D4, a variable heavy chain and variable light chain of 18D8, a variable heavy chain and variable light chain of Hu119-122, a variable heavy chain and variable light chain of Hu106-222, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table OO, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[00649] In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising an OX40L sequence. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:102. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a soluble OX40L sequence. In an

embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:103. In an embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:104.

[00650] In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the V_H and V_L sequences given in Table 18, wherein the V_H and V_L domains are connected by a linker.

TABLE 18: Additional polypeptide domains useful as OX40 binding domains in fusion proteins (*e.g.*, structures I-A and I-B) or as scFv OX40 agonist antibodies.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:102 OX40L	MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL QVSHRYPRIQ SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYFS QEVNISLHYQ KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF CVL	60 120 180 183

SEQ ID NO:103 OX40L soluble domain	SHRYPRIQSI KVQFTEYKKE KGFILTSQKE DEIMKVQNNS VIINCDGFYL ISLKGYFSQE VNISLHYQKD EEPLFQLKKV RSVNSLMVAS LTYKDKVYLN VTTDNTSLDD FHVNGGELIL IHQNPGEFCV L	60 120 131
SEQ ID NO:104 OX40L soluble domain (alternative)	YPRIQSIKVQ FTEYKKEKGF ILTSQKEDEI MKVQNNSVII NCDGFYLISL KGYFSQEVNI SLHYQKDEEP LFQLKKVRSV NSLMVASLTY KDKVYLNVTT DNTSLDDFHV NGGELILIHQ NPGEFCVL	60 120 128
SEQ ID NO:105 variable heavy chain for 008	EVQLVESGGG LVQPGGSLRL SCAASGFTFS NYTMNWVRQA PGKGLEWVSA ISGSGGSTYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YSQVHYALDY WGQGTLVTVS	60 120
SEQ ID NO:106 variable light chain for 008	DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK	60 108
SEQ ID NO:107 variable heavy chain for 011	EVQLVESGGG VVQPGRSLRL SCAASGFTFS DYTMNWVRQA PGKGLEWVSS ISGGSTYYAD SRKGRFTISR DNSKNTLYLQ MNNLRAEDTA VYYCARDRYF RQQNAFDYWG QGTLVTVSSA	60 120
SEQ ID NO:108 variable light chain for 011	DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK	60 108
SEQ ID NO:109 variable heavy chain for 021	EVQLVESGGG LVQPRGSLRL SCAASGFTFS SYAMNWVRQA PGKGLEWVAV ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YITLPNALDY WGQGTLVTVS	60 120
SEQ ID NO:110 variable light chain for 021	DIQMTQSPVS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKPGQSPQ LLIYLGSNRA SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYKSNP PTFGQGTK	60 108
SEQ ID NO:111 variable heavy chain for 023	EVQLVESGGG LVHPGGSLRL SCAGSGFTFS SYAMHWVRQA PGKGLEWVSA IGTGGGTYYA DSVMGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCARYDN VMGLYWFDYW GQGTLVTVSS	60 120
SEQ ID NO:112 variable light chain for 023	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPAFGG GTKVEIKR	60 108
SEQ ID NO:113 heavy chain variable region	EVQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY INPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCANYY GSSLSMDYWG QGTSVTVSS	60 119
SEQ ID NO:114 light chain variable region	DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTLPWTFGG GTKLEIKR	60 108
SEQ ID NO:115 heavy chain variable region	EVQLQQSGPE LVKPGASVKI SCKTSGYTFK DYTMHWVKQS HGKSLEWIGG IYPNNGGSTY NQNFKDKATL TVDKSSSTAY MEFRSLTSED SAVYYCARMG YHGPHLDFDV WGAGTTVTVS P	60 120 121
SEQ ID NO:116 light chain variable region	DIVMTQSHKF MSTSLGDRVS ITCKASQDVG AAVAWYQQKP GQSPKLLIYW ASTRHTGVPD RFTGGGSGTD FTLTISNVQS EDLTDYFCQQ YINYPLTFGG GTKLEIKR	60 108
SEQ ID NO:117 heavy chain variable region of humanized antibody	QIQLVQSGPE LKKPGETVKI SCKASGYTFT DYSMHWVKQA PGKGLKWMGW INTETGEPTY ADDFKGRFAF SLETSASTAY LQINNLKNED TATYFCANPY YDYVSYYAMD YWGHGTSVTV SS	60 120 122
SEQ ID NO:118 heavy chain variable region of humanized antibody	QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV SS	60 120 122
SEQ ID NO:119 light chain variable region of humanized antibody	DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK	60 107
SEQ ID NO:120 light chain variable region of humanized antibody	DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK	60 107
SEQ ID NO:121 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGESLKL SCESNEYEFP SHDMSWVRKT PEKRLELVAA INSDGGSTYY PDTMERRFII SRDNTKKTLY LQMSSLRSED TALYYCARHY DDYYAWFAYW GQGTLVTVSA	60 120
SEQ ID NO:122 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS	60 120
SEQ ID NO:123 light chain	DIVLTQSPAS LAVSLGQRAT ISCRASKSVS TSGYSYMHWY QQKPGQPPKL LIYLASNLES GVPARFSGSG SGTDFTLNIH PVEEEDAATY YCQHSRELPL TFGAGTKLEL K	60 111

variable region of humanized antibody		
SEQ ID NO:124 light chain variable region of humanized antibody	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K	60 111
SEQ ID NO:125 heavy chain variable region	MYLGLNYVFI VFLLNGVQSE VKLEESGGGL VQPGGSMKLS CAASGFTFSD AWMDWVRQSP EKGLEWVAEI RSKANNHATY YAESVNGRFT ISRDDSKSSV YLQMNSLRAE DTGIYYCTWG EVFYFDYWGQ GTTLTVSS	60 120 138
SEQ ID NO:126 light chain variable region	MRPSIQFLGL LLFWLHGAQC DIQMTQSPSS LSASLGGKVT ITCKSSQDIN KYIAWYQHKP GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLLTFGAG TKLELK	60 120 126

[00651] In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain wherein each of the soluble OX40 binding domains lacks a stalk region (which contributes to trimerisation and provides a certain distance to the cell membrane, but is not part of the OX40 binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

[00652] In an embodiment, the OX40 agonist is an OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain, (iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein the TNF superfamily cytokine domain is an OX40 binding domain.

[00653] In some embodiments, the OX40 agonist is MEDI6383. MEDI6383 is an OX40 agonistic fusion protein and can be prepared as described in U.S. Patent No. 6,312,700, the disclosure of which is incorporated by reference herein.

[00654] In an embodiment, the OX40 agonist is an OX40 agonistic scFv antibody comprising any of the foregoing V_H domains linked to any of the foregoing V_L domains.

[00655] In an embodiment, the OX40 agonist is Creative Biolabs OX40 agonist monoclonal antibody MOM-18455, commercially available from Creative Biolabs, Inc., Shirley, NY, USA.

[00656] In an embodiment, the OX40 agonist is OX40 agonistic antibody clone Ber-ACT35 commercially available from BioLegend, Inc., San Diego, CA, USA.

H. Optional Cell Viability Analyses

[00657] Optionally, a cell viability assay can be performed after the first expansion (sometimes referred to as the initial bulk expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. Other assays for use in testing viability can include but are not limited to the Alamar blue assay; and the MTT assay.

1. Cell Counts, Viability, Flow Cytometry

[00658] In some embodiments, cell counts and/or viability are measured. The expression of markers such as but not limited CD3, CD4, CD8, and CD56, as well as any other disclosed or described herein, can be measured by flow cytometry with antibodies, for example but not limited to those commercially available from BD Bio-sciences (BD Biosciences, San Jose, CA) using a FACSCanto™ flow cytometer (BD Biosciences). The cells can be counted manually using a disposable c-chip hemocytometer (VWR, Batavia, IL) and viability can be assessed using any method known in the art, including but not limited to trypan blue staining. The cell viability can also be assayed based on USSN 15/863,634, incorporated by reference herein in its entirety.

[00659] In some cases, the bulk TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to REP and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the bulk or REP TIL populations can be subjected to genetic modifications for suitable treatments.

[00660] According to the present disclosure, a method for assaying TILs for viability and/or further use in administration to a subject. In some embodiments, the method for assay tumor infiltrating lymphocytes (TILs) comprises:

(i) obtaining a first population of TILs;

(ii) performing a first expansion by culturing the first population of TILs in a cell culture medium comprising IL-2, and optionally OKT-3, to produce a second population of TILs; and

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

(iv) harvesting, washing, and cryopreserving the third population of TILs;

(v) storing the cryopreserved TILs at a cryogenic temperature;

(vi) thawing the third population of TILs to provide a thawed third population of TILs; and

(vii) performing an additional second expansion of a portion of the thawed third population of TILs by supplementing the cell culture medium of the third population with IL-2, OKT-3, and APCs for an additional expansion period (sometimes referred to as a reREP period) of at least 3 days, wherein the third expansion is performed to obtain a fourth population of TILs, wherein the number of TILs in the fourth population of TILs is compared to the number of TILs in the third population of TILs to obtain a ratio;

(viii) determining based on the ratio in step (vii) whether the thawed population of TILs is suitable for administration to a patient;

(ix) administering a therapeutically effective dosage of the thawed third population of TILs to the patient when the ratio of the number of TILs in the fourth population of TILs to the number of TILs in the third population of TILs is determined to be greater than 5:1 in step (viii).

[00661] In some embodiments, the TILs are assayed for viability after step (vii).

[00662] The present disclosure also provides further methods for assaying TILs. In some embodiments, the disclosure provides a method for assaying TILs comprising:

(i) obtaining a portion of a first population of cryopreserved TILs;

(ii) thawing the portion of the first population of cryopreserved TILs;

(iii) performing a first expansion by culturing the portion of the first population of

TILs in a cell culture medium comprising IL-2, OKT-3, and antigen presenting cells (APCs) for an additional expansion period (sometimes referred to as a reREP period) of at least 3 days, to produce a second population of TILs, wherein the portion from the first population of TILs is compared to the second population of TILs to obtain a ratio of the number of TILs, wherein the ratio of the number of TILs in the second population of TILs to the number of TILs in the portion of the first population of TILs is greater than 5:1;

(iv) determining based on the ratio in step (iii) whether the first population of TILs is suitable for use in therapeutic administration to a patient;

(v) determining the first population of TILs is suitable for use in therapeutic administration when the ratio of the number of TILs in the second population of TILs to the number of TILs in the first population of TILs is determined to be greater than 5:1 in step (iv).

[00663] In some embodiments, the ratio of the number of TILs in the second population of TILs to the number of TILs in the portion of the first population of TILs is greater than 50:1.

[00664] In some embodiments, the method further comprises performing expansion of the entire first population of cryopreserved TILs from step (i) according to the methods as described in any of the embodiments provided herein.

[00665] In some embodiments, the method further comprises administering the entire first population of cryopreserved TILs from step (i) to the patient.

2. Cell Cultures

[00666] In an embodiment, a method for expanding TILs, including those discussed above as well as exemplified in Figure 1, may include using about 5,000 mL to about 25,000 mL of cell medium, about 5,000 mL to about 10,000 mL of cell medium, or about 5,800 mL to about 8,700 mL of cell medium. In some embodiments, the media is a serum free medium. In some embodiments, the media in the first expansion is serum free. In some embodiments, the media in the second expansion is serum free. . In some embodiments, the media in the first expansion and the second are both serum free. In an embodiment, expanding the number of TILs uses no more than one type of cell culture medium. Any suitable cell culture medium may be used, *e.g.*, AIM-V cell medium (L-glutamine, 50 μM streptomycin sulfate, and 10 μM gentamicin sulfate) cell culture medium (Invitrogen, Carlsbad CA). In this regard, the

inventive methods advantageously reduce the amount of medium and the number of types of medium required to expand the number of TIL. In an embodiment, expanding the number of TIL may comprise feeding the cells no more frequently than every third or fourth day. Expanding the number of cells in a gas permeable container simplifies the procedures necessary to expand the number of cells by reducing the feeding frequency necessary to expand the cells.

[00667] In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME).

[00668] In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable container containing cell medium therein; obtaining TILs from the tumor tissue sample; expanding the number of TILs in a second gas permeable container containing cell medium for a duration of about 7 to 14 days, *e.g.*, about 11 days. In some embodiments pre-REP is about 7 to 14 days, *e.g.*, about 11 days. In some embodiments, REP is about 7 to 14 days, *e.g.*, about 11 days.

[00669] In an embodiment, TILs are expanded in gas-permeable containers. Gas-permeable containers have been used to expand TILs using PBMCs using methods, compositions, and devices known in the art, including those described in U.S. Patent Application Publication No. 2005/0106717 A1, the disclosures of which are incorporated herein by reference. In an embodiment, TILs are expanded in gas-permeable bags. In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the Xuri Cell Expansion System W25 (GE Healthcare). In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the WAVE Bioreactor System, also known as the Xuri Cell Expansion System W5 (GE Healthcare). In an embodiment, the cell expansion system includes a gas permeable cell bag with a volume selected from the group consisting of about 100 mL, about 200 mL, about 300 mL, about 400 mL, about 500 mL, about 600 mL, about 700 mL, about 800 mL, about 900 mL, about 1 L, about 2 L, about 3 L, about 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, and about 10 L.

[00670] In an embodiment, TILs can be expanded in G-Rex flasks (commercially available from Wilson Wolf Manufacturing). Such embodiments allow for cell populations to expand from about $5x10^5$ cells/cm^2 to between $10x10^6$ and $30x10^6$ cells/cm^2. In an embodiment this is without feeding. In an embodiment, this is without feeding so long as medium resides at a height of about 10 cm in the G-Rex flask. In an embodiment this is without feeding but with the addition of one or more cytokines. In an embodiment, the cytokine can be added as a bolus without any need to mix the cytokine with the medium. Such containers, devices, and methods are known in the art and have been used to expand TILs, and include those described in U.S. Patent Application Publication No. US 2014/0377739A1, International Publication No. WO 2014/210036 A1, U.S. Patent Application Publication No. us 2013/0115617 A1, International Publication No. WO 2013/188427 A1, U.S. Patent Application Publication No. US 2011/0136228 A1, U.S. Patent No. US 8,809,050 B2, International publication No. WO 2011/072088 A2, U.S. Patent Application Publication No. US 2016/0208216 A1, U.S. Patent Application Publication No. US 2012/0244133 A1, International Publication No. WO 2012/129201 A1, U.S. Patent Application Publication No. US 2013/0102075 A1, U.S. Patent No. US 8,956,860 B2, International Publication No. WO 2013/173835 A1, U.S. Patent Application Publication No. US 2015/0175966 A1, the disclosures of which are incorporated herein by reference. Such processes are also described in Jin *et al.*, *J. Immunotherapy*, **2012**, 35:283-292.

I. Optional Genetic Engineering of TILs

[00671] In some embodiments, the TILs are optionally genetically engineered to include additional functionalities, including, but not limited to, a high-affinity T cell receptor (TCR), *e.g.*, a TCR targeted at a tumor-associated antigen such as MAGE-1, HER2, or NY-ESO-1, or a chimeric antigen receptor (CAR) which binds to a tumor-associated cell surface molecule (*e.g.*, mesothelin) or lineage-restricted cell surface molecule (*e.g.*, CD19).

J. Optional Cryopreservation of TILs

[00672] As discussed above, and exemplified in Steps A through E as provided in Figure 1, cryopreservation can occur at numerous points throughout the TIL expansion process. In some embodiments, the expanded population of TILs after the second expansion (as provided for example, according to Step D of Figure 1) can be cryopreserved. Cryopreservation can be generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in

solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986. In some embodiments, the TILs are cryopreserved in 5% DMSO. In some embodiments, the TILs are cryopreserved in cell culture media plus 5% DMSO. In some embodiments, the TILs are cryopreserved according to the methods provided in Examples F and G.

[00673] When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

K. Closed Systems for TIL Manufacturing

[00674] The present invention provides for the use of closed systems during the TIL culturing process. Such closed systems allow for preventing and/or reducing microbial contamination, allow for the use of fewer flasks, and allow for cost reductions. In some embodiments, the closed system uses two containers.

[00675] Such closed systems are well-known in the art and can be found, for example, at http://www.fda.gov/cber/guidelines.htm and https://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm076779.htm.

[00676] Sterile connecting devices (STCDs) produce sterile welds between two pieces of compatible tubing. This procedure permits sterile connection of a variety of containers and tube diameters. In some embodiments, the closed systems include luer lock and heat sealed systems as described in for example, Example G. In some embodiments, the closed system is accessed via syringes under sterile conditions in order to maintain the sterility and closed nature of the system. In some embodiments, a closed system as described in Example G is employed. In some embodiments, the TILs are formulated into a final product formulation container according to the method described in Example G, section “Final Formulation and Fill”.

[00677] In some embodiments, the closed system uses one container from the time the tumor fragments are obtained until the TILs are ready for administration to the patient or cryopreserving. In some embodiments when two containers are used, the first container is a

closed G-container and the population of TILs is centrifuged and transferred to an infusion bag without opening the first closed G-container. In some embodiments, when two containers are used, the infusion bag is a HypoThermosol-containing infusion bag. A closed system or closed TIL cell culture system is characterized in that once the tumor sample and/or tumor fragments have been added, the system is tightly sealed from the outside to form a closed environment free from the invasion of bacteria, fungi, and/or any other microbial contamination.

[00678] In some embodiments, the reduction in microbial contamination is between about 5% and about 100%. In some embodiments, the reduction in microbial contamination is between about 5% and about 95%. In some embodiments, the reduction in microbial contamination is between about 5% and about 90%. In some embodiments, the reduction in microbial contamination is between about 10% and about 90%. In some embodiments, the reduction in microbial contamination is between about 15% and about 85%. In some embodiments, the reduction in microbial contamination is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 97%, about 98%, about 99%, or about 100%.

[00679] The closed system allows for TIL growth in the absence and/or with a significant reduction in microbial contamination.

[00680] Moreover, pH, carbon dioxide partial pressure and oxygen partial pressure of the TIL cell culture environment each vary as the cells are cultured. Consequently, even though a medium appropriate for cell culture is circulated, the closed environment still needs to be constantly maintained as an optimal environment for TIL proliferation. To this end, it is desirable that the physical factors of pH, carbon dioxide partial pressure and oxygen partial pressure within the culture liquid of the closed environment be monitored by means of a sensor, the signal whereof is used to control a gas exchanger installed at the inlet of the culture environment, and the that gas partial pressure of the closed environment be adjusted in real time according to changes in the culture liquid so as to optimize the cell culture environment. In some embodiments, the present invention provides a closed cell culture system which incorporates at the inlet to the closed environment a gas exchanger equipped with a monitoring device which measures the pH, carbon dioxide partial pressure and oxygen partial pressure of the closed environment, and optimizes the cell culture environment by automatically adjusting gas concentrations based on signals from the monitoring device.

[00681] In some embodiments, the pressure within the closed environment is continuously or intermittently controlled. That is, the pressure in the closed environment can be varied by means of a pressure maintenance device for example, thus ensuring that the space is suitable for growth of TILs in a positive pressure state, or promoting exudation of fluid in a negative pressure state and thus promoting cell proliferation. By applying negative pressure intermittently, moreover, it is possible to uniformly and efficiently replace the circulating liquid in the closed environment by means of a temporary shrinkage in the volume of the closed environment.

[00682] In some embodiments, optimal culture components for proliferation of the TILs can be substituted or added, and including factors such as IL-2 and/or OKT3, as well as combination, can be added.

L. Optional Cryopreservation of TILs

[00683] Either the bulk TIL population or the expanded population of TILs can be optionally cryopreserved. In some embodiments, cryopreservation occurs on therapeutic TIL population. In some embodiments, cryopreservation occurs on the TILs harvested after the second expansion. In some embodiments, cryopreservation occurs on the TILs in exemplary Step F of Figure 1. In some embodiments, the TILs are cryopreserved in the infusion bag. In some embodiments, the TILs are cryopreserved prior to placement in an infusion bag. In some embodiments, the TILs are cryopreserved and not placed in an infusion bag. In some embodiments, cryopreservation is performed using a cryopreservation medium. In some embodiments, the cryopreservation media contains dimethylsulfoxide (DMSO). This is generally accomplished by putting the TIL population into a freezing solution, e.g. 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See, Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986.

[00684] When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

[00685] In a preferred embodiment, a population of TILs is cryopreserved using CS10 cryopreservation media (CryoStor 10, BioLife Solutions). In a preferred embodiment, a population of TILs is cryopreserved using a cryopreservation media containing dimethylsulfoxide (DMSO). In a preferred embodiment, a population of TILs is cryopreserved using a 1:1 (vol:vol) ratio of CS10 and cell culture media. In a preferred embodiment, a population of TILs is cryopreserved using about a 1:1 (vol:vol) ratio of CS10 and cell culture media, further comprising additional IL-2.

[00686] As discussed above in Steps A through E, cryopreservation can occur at numerous points throughout the TIL expansion process. In some embodiments, the bulk TIL population after the first expansion according to Step B or the expanded population of TILs after the one or more second expansions according to Step D can be cryopreserved. Cryopreservation can be generally accomplished by placing the TIL population into a freezing solution, *e.g.*, 85% complement inactivated AB serum and 15% dimethyl sulfoxide (DMSO). The cells in solution are placed into cryogenic vials and stored for 24 hours at -80 °C, with optional transfer to gaseous nitrogen freezers for cryopreservation. See Sadeghi, *et al., Acta Oncologica* **2013,** *52,* 978-986.

[00687] When appropriate, the cells are removed from the freezer and thawed in a 37 °C water bath until approximately 4/5 of the solution is thawed. The cells are generally resuspended in complete media and optionally washed one or more times. In some embodiments, the thawed TILs can be counted and assessed for viability as is known in the art.

[00688] In some cases, the Step B TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to Step C and Step D and then cryopreserved after Step D. Similarly, in the case where genetically modified TILs will be used in therapy, the Step B or Step D TIL populations can be subjected to genetic modifications for suitable treatments.

IV. TIL Manufacturing Processes (Embodiments of GEN3 Processes, optionally including Defined Media)

[00689] 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 100MCS 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 500MCS 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 100MCS 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 100MCS 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 100MCS 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 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 5 days.

[00690] 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.

[00691] 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%.

[00692] 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%.

[00693] 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%.

[00694] 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%.

[00695] 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%.

[00696] 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.

[00697] 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.

[00698] 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.

[00699] 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.

[00700] In some embodiments, the rapid second expansion of T cells is performed during a period of up to at or about 11 days.

[00701] 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.

[00702] 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.

[00703] 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.

[00704] 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.

[00705] 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.

[00706] 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.

[00707] 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.

[00708] 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.

[00709] In some embodiments, the T cells are tumor infiltrating lymphocytes (TILs).

[00710] In some embodiments, the T cells are marrow infiltrating lymphocytes (MILs).

[00711] In some embodiments, the T cells are peripheral blood lymphocytes (PBLs).

[00712] In some embodiments, the T cells are obtained from a donor suffering from a cancer.

[00713] In some embodiments, the T cells are TILs obtained from a tumor excised from a patient suffering from a cancer.

[00714] In some embodiments, the T cells are MILs obtained from bone marrow of a patient suffering from a hematologic malignancy.

[00715] 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, ovarian cancer, endometrial cancer, thyroid cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the cancer is selected from the group consisting of melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodments, 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.

[00716] 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 one embodiment, 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.

[00717] 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, ovarian cancer, endometrial cancer, thyroid cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodiments, the cancer is selected from the group consisting of melanoma, ovarian cancer, cervical cancer, non-small-cell lung cancer (NSCLC), lung cancer, bladder cancer, breast cancer, cancer caused by human papilloma virus, head and neck cancer (including head and neck squamous cell carcinoma (HNSCC)), glioblastoma (including GBM), gastrointestinal cancer, renal cancer, and renal cell carcinoma. In some embodments, 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.

[00718] An exemplary TIL process known as process 3 (also referred to herein as GEN3) containing some of these features is depicted in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), and some of the advantages of this embodiment of the present invention over process 2A are described in Figures 1, 2, 30, and 31 (in particular, *e.g.*, Figure 8B and/or Figure 8C). Two embodiments of process 3 are shown in Figures 1 and 30 (in particular, *e.g.*, Figure 8B and/or Figure 8C). Process 2A or Gen 2 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 USSN 62/755,954 filed on November 5, 2018 (116983-5045-PR).

[00719] 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.

[00720] 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 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) 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 1 (in particular, *e.g.,* Figure 1B 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C) 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 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is shortened to 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 7 to 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is 8 to 9 days. In some embodiments, the priming first expansion (for example, an expansion described as Step B in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) is shortened to 7 days and the rapid second expansion (for example, an expansion as described in Step D in Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 8B and/or Figure 8C)) 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 1 (in particular, *e.g.*, Figure 1B 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.

[00721] The "Step" Designations A, B, C, *etc.*, below are in reference to the non-limiting example in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) 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 8B and/or Figure 8C) 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

[00722] 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.

[00723] 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, useful TILs are obtained from malignant melanoma tumors, as these have been reported to have particularly high levels of TILs.

[00724] 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.

[00725] 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 with the enzymes to form a tumor digest reaction mixture.

[00726] In some embodiments, the tumor is reconstituted with the lyophilized enzymes in a sterile buffer. In some embodiments, the buffer is sterile HBSS.

[00727] In some embodiments, the enxyme 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.

[00728] 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.

[00729] 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.

[00730] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 1000 IU/ml DNAse, and 1 mg/ml hyaluronidase.

[00731] In some embodiments, the enzyme mixture comprises 10 mg/ml collagenase, 500 IU/ml DNAse, and 1 mg/ml hyaluronidase.

[00732] 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.

[00733] In some embodiments, fragmentation includes physical fragmentation, including, for example, dissection as well as digestion. In some embodiments, the fragmentation is physical fragmentation. In some embodiments, the fragmentation is dissection. In some

embodiments, the fragmentation is by digestion. In some embodiments, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients. In an embodiment, TILs can be initially cultured from enzymatic tumor digests and tumor fragments obtained from patients.

[00734] 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 8B and/or Figure 8C)). 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.

[00735] 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.

[00736] 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.

[00737] 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.

[00738] 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.

[00739] 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

[00740] 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.

[00741] In some emboidments, 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 of lung and/or non-small cell lung carcinoma (NSCLC).

[00742] 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.

[00743] 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, a lung or liver metastatic lesion, or an intra-abdominal or thoracic lymph node or small biopsy can thereof can be employed.

[00744] In some embodiments, the tumor is a melanoma. In some embodiments, the small biopsy for a melanoma comprises a mole or portion thereof.

[00745] In some embodiments, the small biopsy is a punch biopsy. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin. around a suspicious mole. In some embodiments, the punch biopsy is obtained with a circular blade pressed into the skin, and a round piece of skin is removed. In some embodiments, the small biopsy is a punch biopsy and round portion of the tumor is removed.

[00746] In some embodiments, the small biopsy is an excisional biopsy. In some embodiments, the small biopsy is an excisional biopsy and the entire mole or growth is removed. In some embodiments, the small biopsy is an excisional biopsy and the entire mole or growth is removed along with a small border of normal-appearing skin.

[00747] In some embodiments, the small biopsy is an incisional biopsy. In some embodiments, the small biopsy is an incisional biopsy and only the most irregular part of a mole or growth is taken. In some embodiments, the small biopsy is an incisional biopsy and the incisional biopsy is used when other techniques can't be completed, such as if a suspicious mole is very large.

[00748] In some embodiments, the small biopsy is a lung biopsy. In some embodiments, the small biopsy is obtained by bronchoscopy. Generally, bronchoscopy, the patient is put under anesthesia, and a small tool goes through the nose or mouth, down the throat, and into the bronchial passages, where small tools are used to remove some tissue. In some embodiments, where the tumor or growth cannot be reached via bronchoscopy, a transthoracic needle biopsy can be employed. Generally, for a transthoracic needle biopsy, the patient is also under anesthesia and a needle is inserted through the skin directly into the suspicious spot to remove a small sample of tissue. In some embodiments, a transthoracic needle biopsy may require interventional radiology (for example, the use of x-rays or CT scan to guide the needle). In some embodiments, the small biopsy is obtained by needle biopsy. In some embodiments, the small biopsy is obtained endoscopic ultrasound (for example, an endoscope with a light and is placed through the mouth into the esophagus). In some embodiments, the small biopsy is obtained surgically.

[00749] In some embodiments, the small biopsy is a head and neck biopsy. In some embodiments, the small biopsy is an incisional biopsy. In some embodiments, the small biopsy is an incisional biopsy, wherein a small piece of tissue is cut from an abnormal-looking area. In some embodiments, if the abnormal region is easily accessed, the sample may be taken without hospitalization. In some embodiments, if the tumor is deeper inside the mouth or throat, the biopsy may need to be done in an operating room, with general anesthesia. In some embodiments, the small biopsy is an excisional biopsy. In some embodiments, the small biopsy is an excisional biopsy, wherein the whole area is removed. In some embodiments, the small biopsy is a fine needle aspiration (FNA). In some embodiments, the small biopsy is a fine needle aspiration (FNA), wherein a very thin needle attached to a syringe is used to extract (aspirate) cells from a tumor or lump. In some embodiments, the small biopsy is a punch biopsy. In some embodiments, the small biopsy is a punch biopsy, wherein punch forceps are used to remove a piece of the suspicious area.

[00750] In some embodiments, the small biopsy is a cervical biopsy. In some embodiments, the small biopsy is obtained via colposcopy. Generally, colposcopy methods employ the use of a lighted magnifying instrument attached to magnifying binoculars (a colposcope) which is then used to biopsy a small section of the surface of the cervix. In some embodiments, the small biopsy is a conization/cone biopsy. In some embodiments, the small biopsy is a conization/cone biopsy, wherein an outpatient surgery may be needed to remove a larger piece of tissue from the cervix. In some embodiments, the cone biopsy, in addition to helping to confirm a diagnosis, a cone biopsy can serve as an initial treatment.

[00751] The term “solid tumor” refers to an abnormal mass of tissue that usually does not contain cysts or liquid areas. Solid tumors may be benign or malignant. The term “solid tumor cancer refers to malignant, neoplastic, or cancerous solid tumors. Solid tumor cancers include cancers of the lung. In some embodiments, the cancer is non-small cell lung carcinoma (NSCLC). The tissue structure of solid tumors includes interdependent tissue compartments including the parenchyma (cancer cells) and the supporting stromal cells in which the cancer cells are dispersed and which may provide a supporting microenvironment.

[00752] In some embodiments, the sample from the tumor is obtained as a fine needle aspirate (FNA), a core biopsy, a small biopsy (including, for example, a punch biopsy). In some embodiments, sample is placed first into a G-Rex 10. In some embodiments, sample is placed first into a G-Rex 10 when there are 1 or 2 core biopsy and/or small biopsy samples. In some embodiments, sample is placed first into a G-Rex 100 when there are 3, 4, 5, 6, 8, 9,

or 10 or more core biopsy and/or small biopsy samples. In some embodiments, sample is placed first into a G-Rex 500 when there are 3, 4, 5, 6, 8, 9, or 10 or more core biopsy and/or small biopsy samples.

[00753] The FNA can be obtained from a lung tumor, including, for example, an NSCLC. In some embodiments, the FNA is obtained from a lung tumor, such as a lung tumor from a patient with non-small cell lung cancer (NSCLC). In some cases, the patient with NSCLC has previously undergone a surgical treatment.

[00754] TILs described herein can be obtained from an FNA sample. In some cases, the FNA sample is obtained or isolated from the patient using a fine gauge needle ranging from an 18 gauge needle to a 25 gauge needle. The fine gauge needle can be 18 gauge, 19 gauge, 20 gauge, 21 gauge, 22 gauge, 23 gauge, 24 gauge, or 25 gauge. In some embodiments, the FNA sample from the patient can contain at least 400,000 TILs, *e.g.*, 400,000 TILs, 450,000 TILs, 500,000 TILs, 550,000 TILs, 600,000 TILs, 650,000 TILs, 700,000 TILs, 750,000 TILs, 800,000 TILs, 850,000 TILs, 900,000 TILs, 950,000 TILs, or more.

[00755] In some cases, the TILs described herein are obtained from a core biopsy sample. In some cases, the core biopsy sample is obtained or isolated from the patient using a surgical or medical needle ranging from an 11 gauge needle to a 16 gauge needle. The needle can be 11 gauge, 12 gauge, 13 gauge, 14 gauge, 15 gauge, or 16 gauge. In some embodiments, the core biopsy sample from the patient can contain at least 400,000 TILs, *e.g.*, 400,000 TILs, 450,000 TILs, 500,000 TILs, 550,000 TILs, 600,000 TILs, 650,000 TILs, 700,000 TILs, 750,000 TILs, 800,000 TILs, 850,000 TILs, 900,000 TILs, 950,000 TILs, or more.

[00756] In general, the harvested cell suspension is called a "primary cell population" or a "freshly harvested" cell population.

[00757] In some embodiments, the TILs are not obtained from tumor digests. In some embodiments, the solid tumor cores are not fragmented.

[00758] 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, 2mM 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.

2. Methods of Expanding Peripheral Blood Lymphocytes (PBLs) from Peripheral Blood

[00759] PBL Method 1. In an embodiment of the invention, PBLs are expanded using the processes described herein. In an embodiment of the invention, the method comprises obtaining a PBMC sample from whole blood. In an embodiment, the method comprises enriching T-cells by isolating pure T-cells from PBMCs using negative selection of a non-CD19+ fraction. In an embodiment, the method comprises enriching T-cells by isolating pure T-cells from PBMCs using magnetic bead-based negative selection of a non-CD19+ fraction.

[00760] In an embodiment of the invention, PBL Method 1 is performed as follows: On Day 0, a cryopreserved PBMC sample is thawed and PBMCs are counted. T-cells are isolated using a Human Pan T-Cell Isolation Kit and LS columns (Miltenyi Biotec).

[00761] PBL Method 2. In an embodiment of the invention, PBLs are expanded using PBL Method 2, which comprises obtaining a PBMC sample from whole blood. The T-cells from the PBMCs are enriched by incubating the PBMCs for at least three hours at 37°C and then isolating the non-adherent cells.

[00762] In an embodiment of the invention, PBL Method 2 is performed as follows: On Day 0, the cryopreserved PMBC sample is thawed and the PBMC cells are seeded at 6 million cells per well in a 6 well plate in CM-2 media and incubated for 3 hours at 37 degrees Celsius. After 3 hours, the non-adherent cells, which are the PBLs, are removed and counted.

[00763] PBL Method 3. In an embodiment of the invention, PBLs are expanded using PBL Method 3, which comprises obtaining a PBMC sample from peripheral blood. B-cells are isolated using a CD19+ selection and T-cells are selected using negative selection of the non-CD19+ fraction of the PBMC sample.

[00764] In an embodiment of the invention, PBL Method 3 is performed as follows: On Day 0, cryopreserved PBMCs derived from peripheral blood are thawed and counted. CD19+ B-cells are sorted using a CD19 Multisort Kit, Human (Miltenyi Biotec). Of the non-CD19+ cell fraction, T-cells are purified using the Human Pan T-cell Isolation Kit and LS Columns (Miltenyi Biotec).

[00765] In an embodiment, PBMCs are isolated from a whole blood sample. In an embodiment, the PBMC sample is used as the starting material to expand the PBLs. In an embodiment, the sample is cryopreserved prior to the expansion process. In another embodiment, a fresh sample is used as the starting material to expand the PBLs. In an embodiment of the invention, T-cells are isolated from PBMCs using methods known in the art. In an embodiment, the T-cells are isolated using a Human Pan T-cell isolation kit and LS columns. In an embodiment of the invention, T-cells are isolated from PBMCs using antibody selection methods known in the art, for example, CD19 negative selection.

[00766] In an embodiment of the invention, the PBMC sample is incubated for a period of time at a desired temperature effective to identify the non-adherent cells. In an embodiment of the invention, the incubation time is about 3 hours. In an embodiment of the invention, the temperature is about 37° Celsius. The non-adherent cells are then expanded using the process described above.

[00767] In some embodiments, the PBMC sample is from a subject or patient who has been optionally pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor. In some embodiments, the tumor sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor. In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor, has undergone treatment for at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, or 1 year or more. In another embodiment, the PBMCs are derived from a patient who is currently on an ITK inhibitor regimen, such as ibrutinib.

[00768] In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor and is refractory to treatment with a kinase inhibitor or an ITK inhibitor, such as ibrutinib.

[00769] In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor but is no longer

undergoing treatment with a kinase inhibitor or an ITK inhibitor. In some embodiments, the PBMC sample is from a subject or patient who has been pre-treated with a regimen comprising a kinase inhibitor or an ITK inhibitor but is no longer undergoing treatment with a kinase inhibitor or an ITK inhibitor and has not undergone treatment for at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, or at least 1 year or more. In another embodiment, the PBMCs are derived from a patient who has prior exposure to an ITK inhibitor, but has not been treated in at least 3 months, at least 6 months, at least 9 months, or at least 1 year.

[00770] In an embodment of the invention, at Day 0, cells are selected for CD19+ and sorted accordingly. In an embodiment of the invention, the selection is made using antibody binding beads. In an embodiment of the invention, pure T-cells are isolated on Day 0 from the PBMCs.

[00771] In an embodiment of the invention, for patients that are not pre-treated with ibrutinib or other ITK inhibitor, 10-15ml of Buffy Coat will yield about 5×10^9 PBMC, which, in turn, will yield about 5.5×10^7 PBLs.

[00772] In an embodiment of the invention, for patients that are pre-treated with ibrutinib or other ITK inhibitor, the expansion process will yield about 20×10^9 PBLs. In an embodiment of the invention, 40.3×10^6 PBMCs will yield about 4.7×10^5 PBLs.

[00773] In any of the foregoing embodiments, PBMCs may be derived from a whole blood sample, by apheresis, from the buffy coat, or from any other method known in the art for obtaining PBMCs.

3. Methods of Expanding Marrow Infiltrating Lymphocytes (MILs) from PBMCs Derived from Bone Marrow

[00774] MIL Method 3. In an embodiment of the invention, the method comprises obtaining PBMCs from the bone marrow. On Day 0, the PBMCs are selected for CD3+/CD33+/CD20+/CD14+ and sorted, and the non-CD3+/CD33+/CD20+/CD14+ cell fraction is sonicated and a portion of the sonicated cell fraction is added back to the selected cell fraction.

[00775] In an embodiment of the invention, MIL Method 3 is performed as follows: On Day 0, a cryopreserved sample of PBMCs is thawed and PBMCs are counted. The cells are stained with CD3, CD33, CD20, and CD14 antibodies and sorted using a S3e cell sorted (Bio-Rad). The cells are sorted into two fractions – an immune cell fraction (or the MIL fraction) (CD3+CD33+CD20+CD14+) and an AML blast cell fraction (non-CD3+CD33+CD20+CD14+).

[00776] In an embodiment of the invention, PBMCs are obtained from bone marrow. In an embodiment, the PBMCs are obtained from the bone marrow through apheresis, aspiration, needle biopsy, or other similar means known in the art. In an embodiment, the PBMCs are fresh. In another embodiment, the PBMCs are cryopreserved.

[00777] In an embodiment of the invention, MILs are expanded from 10-50 ml of bone marrow aspirate. In an embodiment of the invention, 10ml of bone marrow aspirate is obtained from the patient. In another embodiment, 20ml of bone marrow aspirate is obtained from the patient. In another embodiment, 30ml of bone marrow aspirate is obtained from the patient. In another embodiment, 40ml of bone marrow aspirate is obtained from the patient. In another embodiment, 50ml of bone marrow aspirate is obtained from the patient.

[00778] In an embodiment of the invention, the number of PBMCs yielded from about 10-50ml of bone marrow aspirate is about 5×10^7 to about 10×10^7 PBMCs. In another embodiment, the number of PMBCs yielded is about 7×10^7 PBMCs.

[00779] In an embodiment of the invention, about 5×10^7 to about 10×10^7 PBMCs, yields about 0.5×10^6 to about 1.5×10^6 MILs. In an embodiment of the invention, about 1×10^6 MILs is yielded.

[00780] In an embodiment of the invention, 12×10^6 PBMC derived from bone marrow aspirate yields approximately 1.4×10^5 MILs.

[00781] In any of the foregoing embodiments, PBMCs may be derived from a whole blood sample, from bone marrow, by apheresis, from the buffy coat, or from any other method known in the art for obtaining PBMCs.

B. STEP B: Priming First Expansion

[00782] In some embodiments, the present methods provide for younger TILs, which 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 Donia, at al., *Scandinavian Journal of Immunology,* 75:157–167 (2012); Dudley et al., *Clin Cancer Res,* 16:6122-6131 (2010); Huang et al., *J Immunother*, 28(3):258–267 (2005); Besser et al., *Clin Cancer Res*, 19(17):OF1-OF9 (2013); Besser et al., *J Immunother* 32:415–423 (2009); Robbins, et al., *J Immunol* 2004; 173:7125-7130; Shen et al., J Immunother, 30:123–129 (2007); Zhou, et al., *J Immunother*, 28:53–62 (2005); and Tran, et al., J Immunother, 31:742–751 (2008), all of which are incorporated herein by reference in their entireties.

[00783] After dissection or digestion of tumor fragments and/or tumor fragments, for example such as described in Step A of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), the resulting cells are cultured in serum containing IL-2, OKT-3, and feeder cells (*e.g.*, antigen-presenting feeder cells), under conditions that favor the growth of TILs over tumor and other cells. In some embodiments, the IL-2, OKT-3, and feeder cells are added at culture initiation along with the tumor digest and/or tumor fragments (*e.g.*, at Day 0). In some embodiments, the tumor digests and/or tumor fragments are incubated in a container with up to 60 fragments per container and with 6000 IU/mL of IL-2. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, priming first expansion occurs for a period of 1 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, priming first expansion occurs for a period of 1 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of 5 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of 5 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 6 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 6 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 7 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period

of about 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, this priming first expansion occurs for a period of about 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells.

[00784] In a preferred embodiment, expansion of TILs may be performed using a priming first expansion step (for example such as those described in Step B of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), which can include processes referred to as pre-REP or priming REP and which contains feeder cells from Day 0 and/or from culture initiation) as described below and herein, followed by a rapid 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. In some embodiments, the tumor fragment is between about 1 mm^3 and 10 mm^3.

[00785] 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.

[00786] In some embodiments, there are less than or equal to 240 tumor fragments. In some embodiments, there are less than or equal to 240 tumor fragments placed in less than or equal to 4 containers. In some embodiments, the containers are GREX100 MCS flasks. In some embodiments, less than or equal to 60 tumor fragments are placed in 1 container. In some embodiments, each container comprises less than or equal to 500 mL of media per container. In some embodiments, the media comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media comprises antigen-presenting feeder cells (also referred to herein as "antigen-presenting cells"). In some embodiments, the media comprises 2.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media comprises OKT-3. In some embodiments, the media comprises 30 ng/mL of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng of OKT-3, and 2.5×10^8 antigen-presenting feeder cells. In some embodiments, the media comprises 6000 IU/mL of IL-2, 30 ng/mL of OKT-3, and 2.5×10^8 antigen-presenting feeder cells per container.

[00787] After preparation of the tumor fragments, the resulting cells (*i.e.*, fragments which is a primary cell population) are cultured in media containing IL-2, antigen-presenting feeder cells and OKT-3 under conditions that favor the growth of TILs over tumor and other cells and which allow for TIL priming and accelerated growth from initiation of the culture on Day 0. In some embodiments, the tumor digests and/or tumor fragments are incubated in with 6000 IU/mL of IL-2, as well as antigen-presenting feeder cells and OKT-3. This primary cell population is cultured for a period of days, generally from 1 to 8 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, the growth media during the priming first expansion comprises IL-2 or a variant thereof, as well as antigen-presenting feeder cells and OKT-3. In some embodiments, this primary cell population is cultured for a period of days, generally from 1 to 7 days, resulting in a bulk TIL population, generally about 1×10^8 bulk TIL cells. In some embodiments, the growth media during the priming first expansion comprises IL-2 or a variant thereof, as well as antigen-presenting feeder cells and OKT-3. In some embodiments, the IL-2 is recombinant human IL-2 (rhIL-2). In some embodiments the IL-2 stock solution has a specific activity of $20\text{-}30\times10^6$ 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 C. In some embodiments, the priming 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 priming 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 priming 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 priming 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 priming first expansion culture media comprises about 6,000 IU/mL of IL-2. In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the priming first expansion cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the priming first expansion cell culture medium further comprises IL-2. In a

preferred embodiment, the priming first expansion cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the priming first expansion cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the priming first expansion 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.

[00788] In some embodiments, priming 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 priming first expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the priming first expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the priming first expansion cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the priming first expansion cell culture medium further comprises IL-15. In a preferred embodiment, the priming first expansion cell culture medium comprises about 180 IU/mL of IL-15.

[00789] In some embodiments, priming 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 priming 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 priming 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 priming 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 priming 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 priming first

expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the priming first expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the priming first expansion cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the priming first expansion cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the priming first expansion cell culture medium comprises about 1 IU/mL of IL-21.

[00790] In an embodiment, the priming first expansion cell culture medium comprises OKT-3 antibody. In some embodiments, the priming first expansion cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the priming first expansion cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 µg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL, between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 15 ng/ml and 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises 30 ng/mL of OKT-3 antibody. In some embodiments, the OKT-3 antibody is muromonab.

TABLE 19: 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 NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:2 Muromonab light chain	QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC	60 120 180 213

[00791] In some embodiments, the priming first expansion cell culture medium comprises one or more TNFRSF agonists in a cell culture medium. In some embodiments, the TNFRSF agonist comprises a 4-1BB agonist. In some embodiments, the TNFRSF agonist is a 4-1BB agonist, and the 4-1BB agonist is selected from the group consisting of urelumab, utomilumab, EU-101, a fusion protein, and fragments, derivatives, variants, biosimilars, and combinations thereof. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 0.1 μg/mL and 100 μg/mL. In some embodiments, the TNFRSF agonist is added at a concentration sufficient to achieve a concentration in the cell culture medium of between 20 μg/mL and 40 μg/mL.

[00792] In some embodiments, in addition to one or more TNFRSF agonists, the priming first expansion cell culture medium further comprises IL-2 at an initial concentration of about 3000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist. In some embodiments, in addition to one or more TNFRSF agonists, the priming first expansion cell culture medium further comprises IL-2 at an initial concentration of about 6000 IU/mL and OKT-3 antibody at an initial concentration of about 30 ng/mL, and wherein the one or more TNFRSF agonists comprises a 4-1BB agonist.

[00793] In some embodiments, the priming first expansion culture medium is referred to as "CM", an abbreviation for culture media. In some embodiments, it is referred to as CM1 (culture medium 1). In some embodiments, CM consists of RPMI 1640 with GlutaMAX, supplemented with 10% human AB serum, 25 mM Hepes, and 10 mg/mL gentamicin. In some embodiments, the CM is the CM1 described in the Examples, *see*, Example A. In some embodiments, the priming first expansion occurs in an initial cell culture medium or a first cell culture medium. In some embodiments, the priming first expansion culture medium or the initial cell culture medium or the first cell culture medium comprises IL-2, OKT-3 and antigen-presenting feeder cells (also referred to herein as feeder cells).

[00794] In some embodiments, the culture medium used in the expansion processes disclosed herein is a serum-free medium or a defined medium. In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or a serum replacement. In some embodiments, the serum-free or defined medium is used to prevent and/or decrease experimental variation due in part to the lot-to-lot variation of serum-containing media.

[00795] In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or serum replacement. In some embodiments, the basal cell medium includes, but is not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium , CTS™ OpTmizer™ T-Cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00796] In some embodiments, the serum supplement or serum replacement includes, but is not limited to one or more of CTS™ OpTmizer T-Cell Expansion Serum Supplement, CTS™ Immune Cell Serum Replacement, one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more antibiotics, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^{+}, Al^{3+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Ge^{4+}, Se^{4+}, Br, T, Mn^{2+}, P, Si^{4+}, V^{5+}, Mo^{6+}, Ni^{2+}, Rb^{+}, Sn^{2+} and Zr^{4+}. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[00797] In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00798] In some embodiments, the total serum replacement concentration (vol%) in the serum-free or defined medium is from about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% by volume of the total serum-free

or defined medium. In some embodiments, the total serum replacement concentration is about 3% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 5% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 10% of the total volume of the serum-free or defined medium.

[00799] In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific). In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific) and the final concentration of 2-mercaptoethanol in the media is 55μM.

[00800] In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine, and further comprises about 1000 IU/mL to about 8000 IU/mL of IL-2. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR)

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

[00801] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

[00802] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about

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

[00803] In some embodiments, the defined media described in International PCT Publication No. WO/1998/030679, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum- free culture. The serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^{+}, Al^{3+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Ge^{4+}, Se^{4+}, Br, T, Mn^{2+}, P, Si^{4+}, V^{5+}, Mo^{6+}, Ni^{2+}, Rb^{+}, Sn^{2+} and Zr^{4+}. In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00804] In some embodiments, the concentration of glycine in the defined medium is in the range of from about 5-200 mg/L, the concentration of L- histidine is about 5-250 mg/L, the concentration of L-isoleucine is about 5-300 mg/L, the concentration of L-methionine is about 5-200 mg/L, the concentration of L-phenylalanine is about 5-400 mg/L, the concentration of L-proline is about 1-1000 mg/L, the concentration of L- hydroxyproline is about 1-45 mg/L, the concentration of L-serine is about 1-250 mg/L, the concentration of L-threonine is about 10-500 mg/L, the concentration of L-tryptophan is about 2-110 mg/L, the concentration of L-tyrosine is about 3-175 mg/L, the concentration of L-valine is about 5-500 mg/L, the concentration of thiamine is about 1-20 mg/L, the concentration of reduced glutathione is about 1-20 mg/L, the concentration of L-ascorbic acid-2-phosphate is about 1-200 mg/L, the concentration of iron saturated transferrin is about 1-50 mg/L, the concentration of insulin is about 1-100 mg/L, the concentration of sodium selenite is about 0.000001-0.0001 mg/L, and the concentration of albumin (e.g., AlbuMAX® I) is about 5000-50,000 mg/L.

[00805] In some embodiments, the non-trace element moiety ingredients in the defined medium are present in the concentration ranges listed in the column under the heading "Concentration Range in 1X Medium" in Table A below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading "A Preferred Embodiment of the 1X Medium" in Table A below. In other embodiments, the defined medium is a basal cell medium comprising a serum free supplement. In some of these embodiments, the serum free supplement comprises non-trace moiety ingredients of the type and in the concentrations listed in the column under the heading "A Preferred Embodiment in Supplement" in Table A below.

Table A: Concentrations of Non-Trace Element Moiety Ingredients

Ingredient	A preferred embodiment in supplement (mg/L) (About)	Concentration range in 1X medium (mg/L) (About)	A preferred embodiment in 1X medium (mg/L) (About)
Glycine	150	5-200	53
L-Histidine	940	5-250	183
L-Isoleucine	3400	5-300	615
L-Methionine	90	5-200	44
L-Phenylalanine	1800	5-400	336
L-Proline	4000	1-1000	600
L-Hydroxyproline	100	1-45	15

L-Serine	800	1-250	162
L-Threonine	2200	10-500	425
L-Tryptophan	440	2-110	82
L-Tyrosine	77	3-175	84
L-Valine	2400	5-500	454
Thiamine	33	1-20	9
Reduced Glutathione	10	1-20	1.5
Ascorbic Acid-2-PO_4 (Mg Salt)	330	1-200	50
Transferrin (iron saturated)	55	1-50	8
Insulin	100	1-100	10
Sodium Selenite	0.07	0.000001-0.0001	0.00001
AlbuMAX®I	83,000	5000-50,000	12,500

[00806] In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μM), 2-mercaptoethanol (final concentration of about 100 μM).

[00807] In some embodiments, the defined media described in Smith, *et al.*, "Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement," *Clin Transl Immunology*, 4(1) 2015 (doi: 10.1038/cti.2014.31) are useful in the present invention. Briefly, RPMI or CTS™ OpTmizer™ was used as the basal cell medium, and supplemented with either 0, 2%, 5%, or 10% CTS™ Immune Cell Serum Replacement.

[00808] In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME or βME; also known as 2-mercaptoethanol, CAS 60-24-2).

[00809] In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.*, Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 1 to 8 days, as discussed in the examples and figures. In some embodiments, the priming

first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 2 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 3 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 4 to 8 days, as discussed in the examples and figures. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 1 to 7 days, as discussed in the examples and figures. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 2 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 2 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 3 to 8 days.In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 3 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 4 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 4 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process

is 5 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 5 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 6 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those described in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 6 to 7 days. In some embodiments, the priming first expansion (including processes such as for example those provided in Step B of Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 7 to 8 days. In some embodiments, the priming first expansion (including processes such as for example those provided in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 8 days.In some embodiments, the priming first expansion (including processes such as for example those provided in Step B of Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), which can include those sometimes referred to as the pre-REP or priming REP) process is 7 days.

[00810] In some embodiments, the priming first TIL expansion can proceed for 1 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 1 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 2 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 2 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 3 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 3 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the priming first TIL expansion can proceed for 4 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is

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

[00811] In some embodiments, the priming first expansion of the TILs can proceed for 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days. In some embodiments, the first TIL expansion can proceed for 1 day to 8 days. In some embodiments, the first TIL expansion can proceed for 1 day to 7 days. In some embodiments, the first TIL expansion can proceed for 2 days to 8 days. In some embodiments, the first TIL expansion can proceed for 2 days to 7 days. In some embodiments, the first TIL expansion can proceed for 3 days to 8 days. In some embodiments, the first TIL expansion can proceed for 3 days to 7 days. In some embodiments, the first TIL expansion can proceed for 4 days to 8 days. In some embodiments, the first TIL expansion can proceed for 4 days to 7 days. In some embodiments, the first TIL expansion can proceed for 5 days to 8 days. In some embodiments, the first TIL expansion can proceed for 5 days to 7 days. In some embodiments, the first TIL expansion can proceed for 6 days to 8 days. In some embodiments, the first TIL expansion can proceed for 6 days to 7 days. In some embodiments, the first TIL expansion can proceed for 7 to 8 days. In some embodiments, the first TIL expansion can proceed for 8 days. In some embodiments, the first TIL expansion can proceed for 7 days.

[00812] In some embodiments, a combination of IL-2, IL-7, IL-15, and/or IL-21 are employed as a combination during the priming first expansion. In some embodiments, IL-2, IL-7, IL-15, and/or IL-21 as well as any combinations thereof can be included during the priming first expansion, including, for example during Step B processes according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), 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 priming 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 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) and as described herein.

[00813] In some embodiments, the priming first expansion, for example, Step B according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-10 or a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-10.

1. Feeder Cells and Antigen Presenting Cells

[00814] In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 1 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 4-8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time

during days 4-7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 5-8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 5-7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 6-8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during days 6-7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during day 7 or 8. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP) does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during day 7. In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.,* Figure 8B and/or Figure 8C), as well as those referred to as pre-REP or priming REP)

does not require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion, but rather are added during the priming first expansion at any time during day 8

[00815] In an embodiment, the priming first expansion procedures described herein (for example including expansion such as those described in Step B from Figure 8 (in particular, *e.g.*, Figure 8B), as well as those referred to as pre-REP or priming REP) require feeder cells (also referred to herein as "antigen-presenting cells") at the initiation of the TIL expansion and during the priming first expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In some embodiments, 2.5×10^8 feeder cells are used during the priming first expansion. In some embodiments, 2.5×10^8 feeder cells per container are used during the priming first expansion. In some embodiments, 2.5×10^8 feeder cells per GREX-10 are used during the priming first expansion. In some embodiments, 2.5×10^8 feeder cells per GREX-100 are used during the priming first expansion.

[00816] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

[00817] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells on day 14 is less than the initial viable cell number put into culture on day 0 of the priming first expansion.

[00818] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 have not increased from the initial viable cell number put into culture on day 0 of the priming first expansion. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 3000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 6000 IU/ml IL-2.

[00819] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of

viable cells, cultured in the presence of OKT3 and IL-2, on day 7 have not increased from the initial viable cell number put into culture on day 0 of the priming first expansion. In some embodiments, the PBMCs are cultured in the presence of 5-60 ng/mL OKT3 antibody and 1000-6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 10-50 ng/ml OKT3 antibody and 2000-5000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 20-40 ng/mL OKT3 antibody and 2000-4000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 25-35 ng/mL OKT3 antibody and 2500-3500 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 6000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 15 ng/mL OKT3 antibody and 3000 IU/mL IL-2. In some embodiments, the PBMCs are cultured in the presence of 15 ng/mL OKT3 antibody and 6000 IU/ml IL-2.

[00820] In some embodiments, the antigen-presenting feeder cells are PBMCs. In some embodiments, the antigen-presenting feeder cells are artificial antigen-presenting feeder cells. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

[00821] In an embodiment, the priming first expansion procedures described herein require a ratio of about 2.5×10^8 feeder cells to about 100×10^6 TILs. In another embodiment, the priming first expansion procedures described herein require a ratio of about 2.5×10^8 feeder cells to about 50×10^6 TILs. In yet another embodiment, the priming first expansion described herein require about 2.5×10^8 feeder cells to about 25×10^6 TILs. In yet another embodiment, the priming first expansion described herein require about 2.5×10^8 feeder cells. In yet another embodiment, the priming first expansion requires one-fourth, one-third, five-twelfths, or one-half of the number of feeder cells used in the rapid second expansion.

[00822] In some embodiments, the media in the priming first expansion comprises IL-2. In some embodiments, the media in the priming first expansion comprises 6000 IU/mL of IL-2. In some embodiments, the media in the priming first expansion comprises antigen-presenting feeder cells. In some embodiments, the media in the priming first expansion comprises 2.5 ×

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

[00823] In an embodiment, the priming first expansion procedures described herein require an excess of feeder cells over TILs during the second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In an embodiment, artificial antigen-presenting (aAPC) cells are used in place of PBMCs.

[00824] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

[00825] In an embodiment, artificial antigen presenting cells are used in the priming first expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

[00826] 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.

[00827] Alternatively, using combinations of cytokines for the priming first expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in International Publication No. WO 2015/189356 and WO

2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21, and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

TABLE 20: 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 EEELKPLEEV LNLAQSKNFH LRPRDLISNI NVIVLELKGS ETTFMCEYAD ETATIVEFLN RWITFCQSII STLT	60 120 134
SEQ ID NO:4 Aldesleukin	PTSSSTKKTQ LQLEHLLLDL QMILNGINNY KNPKLTRMLT FKFYMPKKAT ELKHLQCLEE ELKPLEEVLN LAQSKNFHLR PRDLISNINV IVLELKGSET TFMCEYADET ATIVEFLNRW ITFSQSIIST LT	60 120 132
SEQ ID NO:5 recombinant human IL-4 (rhIL-4)	MHKCDITLQE IIKTLNSLTE QKTLCTELTV TDIFAASKNT TEKETFCRAA TVLRQFYSHH EKDTRCLGAT AQQFHRHKQL IRFLKRLDRN LWGLAGLNSC PVKEANQSTL ENFLERLKTI MREKYSKCSS	60 120 130
SEQ ID NO:6 recombinant human IL-7 (rhIL-7)	MDCDIEGKDG KQYESVLMVS IDQLLDSMKE IGSNCLNNEF NFFKRHICDA NKEGMFLFRA ARKLRQFLKM NSTGDFDLHL LKVSEGTTIL LNCTGQVKGR KPAALGEAQP TKSLEENKSL KEQKKLNDLC FLKRLLQEIK TCWNKILMGT KEH	60 120 153
SEQ ID NO:7 recombinant human IL-15 (rhIL-15)	MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS	60 115
SEQ ID NO:8 recombinant human IL-21 (rhIL-21)	MQDRHMIRMR QLIDIVDQLK NYVNDLVPEF LPAPEDVETN CEWSAFSCFQ KAQLKSANTG NNERIINVSI KKLKRKPPST NAGRRQKHRL TCPSCDSYEK KPPKEFLERF KSLLQKMIHQ HLSSRTHGSE DS	60 120 132

C. STEP C: Priming First Expansion to Rapid Second Expansion Transition

[00828] In some cases, the bulk TIL population obtained from the priming first expansion (which can include expansions sometimes referred to as pre-REP), including, for example the TIL population obtained from for example, Step B as indicated in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), can be subjected to a rapid second expansion (which can include expansions sometimes referred to as Rapid Expansion Protocol (REP)) and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the expanded TIL population from the priming first expansion or the expanded TIL population from the rapid second expansion can be subjected to genetic modifications for suitable treatments prior to the expansion step or after the priming first expansion and prior to the rapid second expansion.

[00829] In some embodiments, the TILs obtained from the priming first expansion (for example, from Step B as indicated in Figure 1 (in particular, *e.g.*, Figure 1B and/or Figure

8C)) are stored until phenotyped for selection. In some embodiments, the TILs obtained from the priming first expansion (for example, from Step B as indicated in Figure 1 (in particular, *e.g.,* Figure 1B and/or Figure 8C)) are not stored and proceed directly to the rapid second expansion. In some embodiments, the TILs obtained from the priming first expansion are not cryopreserved after the priming first expansion and prior to the rapid second expansion. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, or 8 days from when tumor fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs at about 3 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs at about 3 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 4 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 4 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 5 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 5 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 6 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 6 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 7 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs at about 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion

occurs at about 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated.

[00830] In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs at 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 1 day to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 1 day to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 2 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 2 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 3 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the second expansion occurs 3 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 4 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 4 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 5 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 5 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 6 days to 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. . In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 6 days to 8 days from when fragmentation occurs and/or when the

first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 7 days to 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 7 days from when fragmentation occurs and/or when the first priming expansion step is initiated. In some embodiments, the transition from the priming first expansion to the rapid second expansion occurs 8 days from when fragmentation occurs and/or when the first priming expansion step is initiated

[00831] In some embodiments, the TILs are not stored after the primary first expansion and prior to the rapid second expansion, and the TILs proceed directly to the rapid second expansion (for example, in some embodiments, there is no storage during the transition from Step B to Step D as shown in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)). In some embodiments, the transition occurs in closed system, as described herein. In some embodiments, the TILs from the priming first expansion, the second population of TILs, proceeds directly into the rapid second expansion with no transition period.

[00832] In some embodiments, the transition from the priming first expansion to the rapid second expansion, for example, Step C according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), 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 GREX-10 or a GREX-100. In some embodiments, the closed system bioreactor is a single bioreactor. In some embodiments, the transition from the priming first expansion to the rapid second expansion involves a scale-up in container size. In some embodiments, the priming first expansion is performed in a smaller container than the rapid second expansion. In some embodiments, the priming first expansion is performed in a GREX-100 and the rapid second expansion is performed in a GREX-500.

D. STEP D: Rapid Second Expansion

[00833] In some embodiments, the TIL cell population is further expanded in number after harvest and the priming first expansion, after Step A and Step B, and the transition referred to as Step C, as indicated in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)). This further expansion is referred to herein as the rapid second expansion, which can include expansion processes generally referred to in the art as a rapid expansion process (Rapid

Expansion Protocol or REP; as well as processes as indicated in Step D of Figure 8 (in particular, *e.g.*, Figure 8B)). The rapid second expansion is generally accomplished using a culture media comprising a number of components, including feeder cells, a cytokine source, and an anti-CD3 antibody, in a gas-permeable container. In some embodiments, 1 day, 2 days, 3 days, or 4 days after initiation of the rapid second expansion (*i.e.*, at days 8, 9, 10, or 11 of the overall Gen 3 process), the TILs are transferred to a larger volume container.

[00834] In some embodiments, the rapid second expansion (which can include expansions sometimes referred to as REP; as well as processes as indicated in Step D of Figure 1 (in particular, *e.g.*, Figure 1B and/or Figure 8C)) of TIL can be performed using any TIL flasks or containers known by those of skill in the art. In some embodiments, the second TIL expansion can proceed for 1 day, 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8 days, 9 days or 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 1 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 1 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 2 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 2 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 3 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 3 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 4 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 4 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 5 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 5 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days to about 10 days

after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days to about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 9 days to about 10 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 1 day after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 2 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 3 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 4 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 5 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 6 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 7 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 8 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 9 days after initiation of the rapid second expansion. In some embodiments, the second TIL expansion can proceed for about 10 days after initiation of the rapid second expansion.

[00835] In an embodiment, the rapid second expansion can be performed in a gas permeable container using the methods of the present disclosure (including, for example, expansions referred to as REP; as well as processes as indicated in Step D of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments, the TILs are expanded in the rapid second expansion in the presence of IL-2, OKT-3, and feeder cells (also referred herein as "antigen-presenting cells"). In some embodiments, the TILs are expanded in the rapid second expansion in the presence of IL-2, OKT-3, and feeder cells, wherein the feeder cells are added to a final concentration that is twice, 2.4 times, 2.5 times, 3 times, 3.5 times or 4 times the concentration of feeder cells present in the priming first expansion. For example, TILs can be rapidly expanded using non-specific T-cell receptor stimulation in the presence of interleukin-2 (IL-2) or interleukin-15 (IL-15). The non-specific T-cell receptor stimulus can include, for example, an anti-CD3 antibody, such as about 30 ng/ml of OKT3, a mouse monoclonal anti-CD3 antibody (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA) or UHCT-1 (commercially available from BioLegend, San

Diego, CA, USA). TILs can be expanded to induce further stimulation of the TILs *in vitro* by including one or more antigens during the second expansion, including antigenic portions thereof, such as epitope(s), of the cancer, which can be optionally expressed from a vector, such as a human leukocyte antigen A2 (HLA-A2) binding peptide, *e.g.*, 0.3 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.

[00836] In an embodiment, the cell culture medium further comprises IL-2. In some embodiments, the cell culture medium comprises about 3000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises about 1000 IU/mL, about 1500 IU/mL, about 2000 IU/mL, about 2500 IU/mL, about 3000 IU/mL, about 3500 IU/mL, about 4000 IU/mL, about 4500 IU/mL, about 5000 IU/mL, about 5500 IU/mL, about 6000 IU/mL, about 6500 IU/mL, about 7000 IU/mL, about 7500 IU/mL, or about 8000 IU/mL of IL-2. In an embodiment, the cell culture medium comprises between 1000 and 2000 IU/mL, between 2000 and 3000 IU/mL, between 3000 and 4000 IU/mL, between 4000 and 5000 IU/mL, between 5000 and 6000 IU/mL, between 6000 and 7000 IU/mL, between 7000 and 8000 IU/mL, or between 8000 IU/mL of IL-2.

[00837] In an embodiment, the cell culture medium comprises OKT-3 antibody. In some embodiments, the cell culture medium comprises about 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 0.1 ng/mL, about 0.5 ng/mL, about 1 ng/mL, about 2.5 ng/mL, about 5 ng/mL, about 7.5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 200 ng/mL, about 500 ng/mL, and about 1 μg/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 0.1 ng/mL and 1 ng/mL, between 1 ng/mL and 5 ng/mL, between 5 ng/mL and 10 ng/mL, between 10 ng/mL and 20 ng/mL,

between 20 ng/mL and 30 ng/mL, between 30 ng/mL and 40 ng/mL, between 40 ng/mL and 50 ng/mL, and between 50 ng/mL and 100 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 15 ng/ml and 30 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises between 30 ng/ml and 60 ng/mL of OKT-3 antibody. In an embodiment, the cell culture medium comprises about 30 ng/mL OKT-3. In an embodiment, the cell culture medium comprises about 60 ng/mL OKT-3. In some embodiments, the OKT-3 antibody is muromonab.

[00838] In some embodiments, the media in the rapid second expansion comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media in the rapid second expansion comprises antigen-presenting feeder cells. In some embodiments, the media in the rapid second expansion comprises 7.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media in the rapid second expansion comprises OKT-3. In some embodiments, the in the rapid second expansion media comprises 500 mL of culture medium and 30 μg of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the in the rapid second expansion media comprises 6000 IU/mL of IL-2, 60 ng/mL of OKT-3, and 7.5×10^8 antigen-presenting feeder cells. In some embodiments, the media comprises 500 mL of culture medium and 6000 IU/mL of IL-2, 30 μg of OKT-3, and 7.5×10^8 antigen-presenting feeder cells per container.

[00839] In some embodiments, the media in the rapid second expansion comprises IL-2. In some embodiments, the media comprises 6000 IU/mL of IL-2. In some embodiments, the media in the rapid second expansion comprises antigen-presenting feeder cells. In some embodiments, the media comprises between 5×10^8 and 7.5×10^8 antigen-presenting feeder cells per container. In some embodiments, the media in the rapid second expansion comprises OKT-3. In some embodiments, the media in the rapid second expansion comprises 500 mL of culture medium and 30 μg of OKT-3 per container. In some embodiments, the container is a GREX100 MCS flask. In some embodiments, the media in the rapid second expansion comprises 6000 IU/mL of IL-2, 60 ng/mL of OKT-3, and between 5×10^8 and 7.5×10^8 antigen-presenting feeder cells. In some embodiments, the media in the rapid second expansion comprises 500 mL of culture medium and 6000 IU/mL of IL-2, 30 μg of OKT-3, and between 5×10^8 and 7.5×10^8 antigen-presenting feeder cells per container.

[00840] 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.

[00841] 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.

[00842] 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 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), 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 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) and as described herein.

[00843] 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).

[00844] In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15, about 400 IU/mL of IL-15, about 300 IU/mL of IL-15, about 200 IU/mL of IL-15, about 180 IU/mL of IL-15, about 160 IU/mL of IL-15, about 140 IU/mL of IL-15,

about 120 IU/mL of IL-15, or about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 500 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 400 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 300 IU/mL of IL-15 to about 100 IU/mL of IL-15. In some embodiments, the second expansion culture media comprises about 200 IU/mL of IL-15. In some embodiments, the cell culture medium comprises about 180 IU/mL of IL-15. In an embodiment, the cell culture medium further comprises IL-15. In a preferred embodiment, the cell culture medium comprises about 180 IU/mL of IL-15.

[00845] In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21, about 15 IU/mL of IL-21, about 12 IU/mL of IL-21, about 10 IU/mL of IL-21, about 5 IU/mL of IL-21, about 4 IU/mL of IL-21, about 3 IU/mL of IL-21, about 2 IU/mL of IL-21, about 1 IU/mL of IL-21, or about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 20 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 15 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 12 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 10 IU/mL of IL-21 to about 0.5 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 5 IU/mL of IL-21 to about 1 IU/mL of IL-21. In some embodiments, the second expansion culture media comprises about 2 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 1 IU/mL of IL-21. In some embodiments, the cell culture medium comprises about 0.5 IU/mL of IL-21. In an embodiment, the cell culture medium further comprises IL-21. In a preferred embodiment, the cell culture medium comprises about 1 IU/mL of IL-21.

[00846] In some embodiments, the antigen-presenting feeder cells (APCs) are PBMCs. In an embodiment, the ratio of TILs to PBMCs and/or antigen-presenting cells in the rapid expansion and/or the second expansion is about 1 to 10, about 1 to 15, about 1 to 20, about 1 to 25, about 1 to 30, about 1 to 35, about 1 to 40, about 1 to 45, about 1 to 50, about 1 to 75, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 50 and 1 to 300. In an embodiment,

the ratio of TILs to PBMCs in the rapid expansion and/or the second expansion is between 1 to 100 and 1 to 200.

[00847] In an embodiment, REP and/or the rapid second expansion is performed in flasks with the bulk TILs being mixed with a 100- or 200-fold excess of inactivated feeder cells, wherein the feeder cell concentration is at least 1.1 times (1.1X), 1.2X, 1.3X, 1.4X, 1.5X, 1.6X, 1.7X, 1.8X, 1.8X, 2X, 2.1X2.2X, 2.3X, 2.4X, 2.5X, 2.6X, 2.7X, 2.8X, 2.9X, 3.0X, 3.1X, 3.2X, 3.3X, 3.4X, 3.5X, 3.6X, 3.7X, 3.8X, 3.9X or 4.0X the feeder cell concentration in the priming first expansion, 30 ng/mL OKT3 anti-CD3 antibody and 6000 IU/mL IL-2 in 150 ml media. Media replacement is done (generally 2/3 media replacement via aspiration of 2/3 of spent media and replacement with an equal volume of fresh media) until the cells are transferred to an alternative growth chamber. Alternative growth chambers include G-REX flasks and gas permeable containers as more fully discussed below.

[00848] In some embodiments, the rapid second expansion (which can include processes referred to as the REP process) is 7 to 9 days, as discussed in the examples and figures. In some embodiments, the second expansion is 7 days. In some embodiments, the second expansion is 8 days. In some embodiments, the second expansion is 9 days.

[00849] In an embodiment, the second expansion (which can include expansions referred to as REP, as well as those referred to in Step D of Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C) 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, 6000 IU per mL of IL-2, and added back to the original GREX-100 flasks. When TIL are expanded serially in GREX-100 flasks, on day 10 or 11 the TILs can be moved to a larger flask, such as a GREX-500. The cells may be harvested on day 14 of culture. The cells may be harvested on day 15 of culture. The cells may be harvested on day 16 of culture. In some embodiments, media replacement is done until the cells are transferred to an alternative growth chamber. In some embodiments, 2/3 of the media is replaced by aspiration of spent media and

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

[00850] In some embodiments, the culture medium used in the expansion processes disclosed herein is a serum-free medium or a defined medium. In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or a serum replacement. In some embodiments, the serum-free or defined medium is used to prevent and/or decrease experimental variation due in part to the lot-to-lot variation of serum-containing media.

[00851] In some embodiments, the serum-free or defined medium comprises a basal cell medium and a serum supplement and/or serum replacement. In some embodiments, the basal cell medium includes, but is not limited to CTS™ OpTmizer™ T-cell Expansion Basal Medium , CTS™ OpTmizer™ T-Cell Expansion SFM, CTS™ AIM-V Medium, CTS™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00852] In some embodiments, the serum supplement or serum replacement includes, but is not limited to one or more of CTS™ OpTmizer T-Cell Expansion Serum Supplement, CTS™ Immune Cell Serum Replacement, one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more antibiotics, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^{+}, Al^{3+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Ge^{4+}, Se^{4+}, Br, T, Mn^{2+}, P, Si^{4+}, V^{5+}, Mo^{6+}, Ni^{2+}, Rb^{+}, Sn^{2+} and Zr^{4+}. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or 2-mercaptoethanol.

[00853] In some embodiments, the CTS™OpTmizer™ T-cell Immune Cell Serum Replacement is used with conventional growth media, including but not limited to CTS™

OpTmizer™ T-cell Expansion Basal Medium, CTS™ OpTmizer™ T-cell Expansion SFM, CTS™ AIM-V Medium, CST™ AIM-V SFM, LymphoONE™ T-Cell Expansion Xeno-Free Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM), Glasgow's Minimal Essential Medium (G-MEM), RPMI growth medium, and Iscove's Modified Dulbecco's Medium.

[00854] In some embodiments, the total serum replacement concentration (vol%) in the serum-free or defined medium is from about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% by volume of the total serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 3% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 5% of the total volume of the serum-free or defined medium. In some embodiments, the total serum replacement concentration is about 10% of the total volume of the serum-free or defined medium.

[00855] In some embodiments, the serum-free or defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM.

[00856] In some embodiments, the defined medium is CTS™ OpTmizer™ T-cell Expansion SFM (ThermoFisher Scientific). Any formulation of CTS™ OpTmizer™ is useful in the present invention. CTS™ OpTmizer™ T-cell Expansion SFM is a combination of 1L CTS™ OpTmizer™ T-cell Expansion Basal Medium and 26 mL CTS™ OpTmizer™ T-Cell Expansion Supplement, which are mixed together prior to use. In some embodiments, the CTS™ OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), along with 2-mercaptoethanol at 55mM. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3% of the CTS™ Immune Cell Serum Replacement (SR) (ThermoFisher Scientific), 55mM of 2-mercaptoethanol, and 2mM of L-glutamine. In some embodiments, the CTS™OpTmizer™ T-cell Expansion SFM is supplemented with about 3%

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

[00857] In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of from about 0.1mM to about 10mM, 0.5mM to about 9mM, 1mM to about 8mM, 2mM to about 7mM, 3mM to about 6mM, or 4mM to about 5 mM. In some embodiments, the serum-free medium or defined medium is supplemented with glutamine (i.e., GlutaMAX®) at a concentration of about 2mM.

[00858] In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of from about 5mM to about 150mM, 10mM to about 140mM, 15mM to about 130mM, 20mM to about 120mM, 25mM to about 110mM, 30mM to about 100mM, 35mM to about 95mM, 40mM to about 90mM, 45mM to about 85mM, 50mM to about 80mM, 55mM to about 75mM, 60mM to about 70mM, or about 65mM. In some embodiments, the serum-free medium or defined medium is supplemented with 2-mercaptoethanol at a concentration of about 55mM.

[00859] In some embodiments, the defined media described in International PCT Publication No. WO/1998/030679, which is herein incorporated by reference, are useful in the present invention. In that publication, serum-free eukaryotic cell culture media are described. The serum-free, eukaryotic cell culture medium includes a basal cell culture medium supplemented with a serum-free supplement capable of supporting the growth of cells in serum- free culture. The serum-free eukaryotic cell culture medium supplement comprises or is obtained by combining one or more ingredients selected from the group consisting of one or more albumins or albumin substitutes, one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, one or more trace elements, and one or more antibiotics. In some embodiments, the defined medium further comprises L-glutamine, sodium bicarbonate and/or beta-mercaptoethanol. In some embodiments, the defined medium comprises an albumin or an albumin substitute and one or more ingredients selected from group consisting of one or more amino acids, one or more vitamins, one or more transferrins or transferrin substitutes, one or more antioxidants, one or more insulins or insulin substitutes, one or more collagen precursors, and one or more trace elements. In some embodiments, the defined medium comprises albumin and one or more ingredients selected from the group consisting of glycine, L- histidine, L-isoleucine, L-methionine, L-phenylalanine, L-proline, L- hydroxyproline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, thiamine, reduced glutathione, L-ascorbic acid-2-phosphate, iron saturated transferrin, insulin, and compounds containing the trace element moieties Ag^{+}, Al^{3+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Ge^{4+}, Se^{4+}, Br, T, Mn^{2+}, P, Si^{4+}, V^{5+}, Mo^{6+}, Ni^{2+}, Rb^{+}, Sn^{2+} and Zr^{4+}. In some embodiments, the basal cell media is selected from the group consisting of Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Basal Medium Eagle (BME), RPMI 1640, F-10, F-12, Minimal Essential Medium (αMEM),

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

[00860] In some embodiments, the concentration of glycine in the defined medium is in the range of from about 5-200 mg/L, the concentration of L- histidine is about 5-250 mg/L, the concentration of L-isoleucine is about 5-300 mg/L, the concentration of L-methionine is about 5-200 mg/L, the concentration of L-phenylalanine is about 5-400 mg/L, the concentration of L-proline is about 1-1000 mg/L, the concentration of L- hydroxyproline is about 1-45 mg/L, the concentration of L-serine is about 1-250 mg/L, the concentration of L-threonine is about 10-500 mg/L, the concentration of L-tryptophan is about 2-110 mg/L, the concentration of L-tyrosine is about 3-175 mg/L, the concentration of L-valine is about 5-500 mg/L, the concentration of thiamine is about 1-20 mg/L, the concentration of reduced glutathione is about 1-20 mg/L, the concentration of L-ascorbic acid-2-phosphate is about 1-200 mg/L, the concentration of iron saturated transferrin is about 1-50 mg/L, the concentration of insulin is about 1-100 mg/L, the concentration of sodium selenite is about 0.000001-0.0001 mg/L, and the concentration of albumin (e.g., AlbuMAX® I) is about 5000-50,000 mg/L.

[00861] In some embodiments, the non-trace element moiety ingredients in the defined medium are present in the concentration ranges listed in the column under the heading "Concentration Range in 1X Medium" in Table A below. In other embodiments, the non-trace element moiety ingredients in the defined medium are present in the final concentrations listed in the column under the heading "A Preferred Embodiment of the 1X Medium" in Table A below. In other embodiments, the defined medium is a basal cell medium comprising a serum free supplement. In some of these embodiments, the serum free supplement comprises non-trace moiety ingredients of the type and in the concentrations listed in the column under the heading "A Preferred Embodiment in Supplement" in Table A below.

Table A: Concentrations of Non-Trace Element Moiety Ingredients

Ingredient	A preferred embodiment in supplement (mg/L) (About)	Concentration range in 1X medium (mg/L) (About)	A preferred embodiment in 1X medium (mg/L) (About)
Glycine	150	5-200	53
L-Histidine	940	5-250	183
L-Isoleucine	3400	5-300	615
L-Methionine	90	5-200	44

L-Phenylalanine	1800	5-400	336
L-Proline	4000	1-1000	600
L-Hydroxyproline	100	1-45	15
L-Serine	800	1-250	162
L-Threonine	2200	10-500	425
L-Tryptophan	440	2-110	82
L-Tyrosine	77	3-175	84
L-Valine	2400	5-500	454
Thiamine	33	1-20	9
Reduced Glutathione	10	1-20	1.5
Ascorbic Acid-2-PO_4 (Mg Salt)	330	1-200	50
Transferrin (iron saturated)	55	1-50	8
Insulin	100	1-100	10
Sodium Selenite	0.07	0.000001-0.0001	0.00001
AlbuMAX®I	83,000	5000-50,000	12,500

[00862] In some embodiments, the osmolarity of the defined medium is between about 260 and 350 mOsmol. In some embodiments, the osmolarity is between about 280 and 310 mOsmol. In some embodiments, the defined medium is supplemented with up to about 3.7 g/L, or about 2.2 g/L sodium bicarbonate. The defined medium can be further supplemented with L-glutamine (final concentration of about 2 mM), one or more antibiotics, non-essential amino acids (NEAA; final concentration of about 100 μM), 2-mercaptoethanol (final concentration of about 100 μM).

[00863] In some embodiments, the defined media described in Smith, *et al.*, "Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement," *Clin Transl Immunology*, 4(1) 2015 (doi: 10.1038/cti.2014.31) are useful in the present invention. Briefly, RPMI or CTS™ OpTmizer™ was used as the basal cell medium, and supplemented with either 0, 2%, 5%, or 10% CTS™ Immune Cell Serum Replacement.

[00864] In an embodiment, the cell medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME or βME; also known as 2-mercaptoethanol, CAS 60-24-2).

[00865] In an embodiment, the rapid second expansion (including expansions referred to as REP) is performed and further comprises a step wherein TILs are selected for superior tumor

reactivity. Any selection method known in the art may be used. For example, the methods described in U.S. Patent Application Publication No. 2016/0010058 A1, the disclosures of which are incorporated herein by reference, may be used for selection of TILs for superior tumor reactivity.

[00866] Optionally, a cell viability assay can be performed after the rapid second expansion (including expansions referred to as the REP expansion), using standard assays known in the art. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. In some embodiments, TIL samples can be counted and viability determined using a Cellometer K2 automated cell counter (Nexcelom Bioscience, Lawrence, MA). In some embodiments, viability is determined according to the standard Cellometer K2 Image Cytometer Automatic Cell Counter protocol.

[00867] 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).

[00868] In some embodiments, the rapid second expansion culture medium (*e.g.*, sometimes referred to as CM2 or the second cell culture medium), comprises IL-2, OKT-3, as well as the antigen-presenting feeder cells (APCs), as discussed in more detail below. In

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

[00869] In some embodiments, the rapid second expansion, for example, Step D according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-100 or a G-REX-500. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-500.

1. Feeder Cells and Antigen Presenting Cells

[00870] In an embodiment, the rapid second expansion procedures described herein (for example including expansion such as those described in Step D from Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), as well as those referred to as REP) require an excess of feeder cells during REP TIL expansion and/or during the rapid second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation.

[00871] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the REP procedures, as described in the examples, which provides an exemplary protocol for evaluating the replication incompetence of irradiate allogeneic PBMCs.

[00872] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of

viable cells on day 7 or 14 is less than the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion).

[00873] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 3000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 60 ng/ml OKT3 antibody and 6000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 60 ng/ml OKT3 antibody and 3000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30 ng/ml OKT3 antibody and 6000 IU/ml IL-2.

[00874] In some embodiments, PBMCs are considered replication incompetent and acceptable for use in the TIL expansion procedures described herein if the total number of viable cells, cultured in the presence of OKT3 and IL-2, on day 7 and day 14 has not increased from the initial viable cell number put into culture on day 0 of the REP and/or day 0 of the second expansion (*i.e.*, the start day of the second expansion). In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 1000-6000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 2000-5000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 2000-4000 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 2500-3500 IU/ml IL-2. In some embodiments, the PBMCs are cultured in the presence of 30-60 ng/ml OKT3 antibody and 6000 IU/ml IL-2.

[00875] In some embodiments, the antigen-presenting feeder cells are PBMCs. In some embodiments, the antigen-presenting feeder cells are artificial antigen-presenting feeder cells. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is about 1 to 10, about 1 to 25, about 1 to 50, about 1 to 100, about 1 to 125, about 1 to 150, about 1 to 175, about 1 to 200, about 1 to 225, about 1 to 250, about 1 to 275, about 1 to 300, about 1 to 325, about 1 to 350, about 1 to 375, about 1 to 400, or about 1 to 500. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 50 and 1 to 300. In an embodiment, the ratio of TILs to antigen-presenting feeder cells in the second expansion is between 1 to 100 and 1 to 200.

[00876] In an embodiment, the second expansion procedures described herein require a ratio of about 5×10^8 feeder cells to about 100×10^6 TILs. In an embodiment, the second expansion procedures described herein require a ratio of about 7.5×10^8 feeder cells to about 100×10^6 TILs. In another embodiment, the second expansion procedures described herein require a ratio of about 5×10^8 feeder cells to about 50×10^6 TILs. In another embodiment, the second expansion procedures described herein require a ratio of about 7.5×10^8 feeder cells to about 50×10^6 TILs. In yet another embodiment, the second expansion procedures described herein require about 5×10^8 feeder cells to about 25×10^6 TILs. In yet another embodiment, the second expansion procedures described herein require about 7.5×10^8 feeder cells to about 25×10^6 TILs. In yet another embodiment, the rapid second expansion requires twice the number of feeder cells as the rapid second expansion. In yet another embodiment, when the priming first expansion described herein requires about 2.5×10^8 feeder cells, the rapid second expansion requires about 5×10^8 feeder cells. In yet another embodiment, when the priming first expansion described herein requires about 2.5×10^8 feeder cells, the rapid second expansion requires about 7.5×10^8 feeder cells. In yet another embodiment, the rapid second expansion requires two times (2.0X), 2.5X, 3.0X, 3.5X or 4.0X the number of feeder cells as the priming first expansion.

[00877] In an embodiment, the rapid second expansion procedures described herein require an excess of feeder cells during the rapid second expansion. In many embodiments, the feeder cells are peripheral blood mononuclear cells (PBMCs) obtained from standard whole blood units from allogeneic healthy blood donors. The PBMCs are obtained using standard methods such as Ficoll-Paque gradient separation. In an embodiment, artificial antigen-presenting (aAPC) cells are used in place of PBMCs. In some embodiments, the PBMCs are added to the rapid second expansion at twice the concentration of PBMCs that were added to the priming first expansion.

[00878] In general, the allogenic PBMCs are inactivated, either via irradiation or heat treatment, and used in the TIL expansion procedures described herein, including the exemplary procedures described in the figures and examples.

[00879] In an embodiment, artificial antigen presenting cells are used in the rapid second expansion as a replacement for, or in combination with, PBMCs.

2. Cytokines

[00880] The rapid second expansion methods described herein generally use culture media with high doses of a cytokine, in particular IL-2, as is known in the art.

[00881] Alternatively, using combinations of cytokines for the rapid second expansion of TILs is additionally possible, with combinations of two or more of IL-2, IL-15 and IL-21 as is generally outlined in WO 2015/189356 and WO 2015/189357, hereby expressly incorporated by reference in their entirety. Thus, possible combinations include IL-2 and IL-15, IL-2 and IL-21, IL-15 and IL-21, and IL-2, IL-15 and IL-21, with the latter finding particular use in many embodiments. The use of combinations of cytokines specifically favors the generation of lymphocytes, and in particular T-cells as described therein.

E. STEP E: Harvest TILS

[00882] After the rapid 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 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments the TILs are harvested after two expansion steps, for example as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C). In some embodiments the TILs are harvested after two expansion steps, one priming first expansion and one rapid second expansion, for example as provided in Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C).

[00883] TILs can be harvested in any appropriate and sterile manner, including, for example by centrifugation. Methods for TIL harvesting are well known in the art and any such known methods can be employed with the present process. In some embodiments, TILS are harvested using an automated system.

[00884] Cell harvesters and/or cell processing systems are commercially available from a variety of sources, including, for example, Fresenius Kabi, Tomtec Life Science, Perkin Elmer, and Inotech Biosystems International, Inc. Any cell based harvester can be employed with the present methods. In some embodiments, the cell harvester and/or cell processing system is a membrane-based cell harvester. In some embodiments, cell harvesting is via a cell processing system, such as the LOVO system (manufactured by Fresenius Kabi). The term "LOVO cell processing system" also refers to any instrument or device manufactured by any vendor that can pump a solution comprising cells through a membrane or filter such as a spinning membrane or spinning filter in a sterile and/or closed system environment, allowing for continuous flow and cell processing to remove supernatant or cell culture media without pelletization. In some embodiments, the cell harvester and/or cell processing system can

perform cell separation, washing, fluid-exchange, concentration, and/or other cell processing steps in a closed, sterile system.

[00885] In some embodiments, the rapid second expansion, for example, Step D according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), is performed in a closed system bioreactor. In some embodiments, a closed system is employed for the TIL expansion, as described herein. In some embodiments, a bioreactor is employed. In some embodiments, a bioreactor is employed as the container. In some embodiments, the bioreactor employed is for example a G-REX-100 or a G-REX-500. In some embodiments, the bioreactor employed is a G-REX-100. In some embodiments, the bioreactor employed is a G-REX-500.

[00886] In some embodiments, Step E according to Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C), 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 herein is employed.

[00887] In some embodiments, TILs are harvested according to the methods described in herein. In some embodiments, TILs between days 14 and 16 are harvested using the methods as described herein. In some embodiments, TILs are harvested at 14 days using the methods as described herein. In some embodiments, TILs are harvested at 15 days using the methods as described herein. In some embodiments, TILs are harvested at 16 days using the methods as described herein.

F. STEP F: Final Formulation/ Transfer to Infusion Bag

[00888] After Steps A through E as provided in an exemplary order in Figure 8 (in particular, *e.g.*, Figure 8B) and as outlined in detailed above and herein are complete, cells are transferred to a container for use in administration to a patient. In some embodiments, once a therapeutically sufficient number of TILs are obtained using the expansion methods described above, they are transferred to a container for use in administration to a patient.

[00889] In an embodiment, TILs expanded using the methods of the present disclosure are administered to a patient as a pharmaceutical composition. In an embodiment, the pharmaceutical composition is a suspension of TILs in a sterile buffer. TILs expanded as disclosed herein may be administered by any suitable route as known in the art. In some embodiments, the TILs are administered as a single intra-arterial or intravenous infusion,

which preferably lasts approximately 30 to 60 minutes. Other suitable routes of administration include intraperitoneal, intrathecal, and intralymphatic.

G. PBMC Feeder Cell Ratios

[00890] In some embodiments, the culture media used in expansion methods described herein (see for example, Figure 8 (in particular, *e.g.*, Figure 8B and/or Figure 8C)) include an anti-CD3 antibody e.g. OKT-3. An anti-CD3 antibody in combination with IL-2 induces T cell activation and cell division in the TIL population. This effect can be seen with full length antibodies as well as Fab and F(ab')2 fragments, with the former being generally preferred; see, *e.g.,* Tsoukas *et al.*, *J. Immunol.* **1985,** *135,* 1719, hereby incorporated by reference in its entirety.

[00891] In an embodiment, the number of PBMC feeder layers is calculated as follows:

A. Volume of a T-cell (10 μm diameter): $V = (4/3)\,\pi r^3 = 523.6\ \mu m^3$

B. Columne of G-Rex 100 (M) with a 40 μm (4 cells) height: $V = (4/3)\,\pi r^3 = 4\times10^{12}\ \mu m^3$

C. Number cell required to fill column B: $4\times10^{12}\ \mu m^3 / 523.6\ \mu m^3 = 7.6\times10^{8}\ \mu m^3 * 0.64 = 4.86\times10^{8}$

D. Number cells that can be optimally activated in 4D space: $4.86\times10^{8} / 24 = 20.25\times10^{6}$

E. Number of feeders and TIL extrapolated to G-Rex 500: TIL: 100×10^{6} and Feeder: 2.5×10^{9}

In this calculation, an approximation of the number of mononuclear cells required to provide an icosahedral geometry for activation of TIL in a cylinder with a 100 cm^2 base is used. The calculation derives the experimental result of ~5×10^{8} for threshold activation of T-cells which closely mirrors NCI experimental data.[(1)] (C) The multiplier (0.64) is the random packing density for equivalent spheres as calculated by Jaeger and Nagel in 1992 [(2)]. (D) The divisor 24 is the number of equivalent spheres that could contact a similar object in 4 dimensional space "the Newton number."[(3)].

[(1)] Jin, Jianjian, et.al., Simplified Method of the Growth of Human Tumor Infiltrating Lymphocytes (TIL) in Gas-Permeable Flasks to Numbers Needed for Patient Treatment. J Immunother. 2012 Apr; 35(3): 283–292.

[(2)] Jaeger HM, Nagel SR. Physics of the granular state. Science. 1992 Mar 20;255(5051):1523-31.

(3) O. R. Musin (2003). "The problem of the twenty-five spheres". Russ. Math. Surv. 58 (4): 794–795.

[00892] In an embodiment, the number of antigen-presenting feeder cells exogenously supplied during the priming first expansion is approximately one-half the number of antigen-presenting feeder cells exogenously supplied during the rapid second expansion. In certain embodiments, the method comprises performing the priming first expansion in a cell culture medium which comprises approximately 50% fewer antigen presenting cells as compared to the cell culture medium of the rapid second expansion.

[00893] In another embodiment, the number of antigen-presenting feeder cells (APCs) exogenously supplied during the rapid second expansion is greater than the number of APCs exogenously supplied during the priming first expansion.

[00894] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 20:1.

[00895] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 10:1.

[00896] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 9:1.

[00897] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 8:1.

[00898] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 7:1.

[00899] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 6:1.

[00900] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 5:1.

[00901] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 4:1.

[00902] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion) is selected from a range of from at or about 1.1:1 to at or about 3:1.

[00903] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.9:1.

[00904] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.8:1.

[00905] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.7:1.

[00906] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.6:1.

[00907] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.5:1.

[00908] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.4:1.

[00909] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.3:1.

[00910] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.2:1.

[00911] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.1:1.

[00912] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2:1.

[00913] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 10:1.

[00914] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 5:1.

[00915] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 4:1.

[00916] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 3:1.

[00917] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.9:1.

[00918] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.8:1.

[00919] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.7:1.

[00920] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.6:1.

[00921] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.5:1.

[00922] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.4:1.

[00923] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.3:1.

[00924] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about about 2:1 to at or about 2.2:1.

[00925] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.1:1.

[00926] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is at or about 2:1.

[00927] In another embodiment, the ratio of the number of APCs exogenously supplied during the rapid second expansion to the number of APCs exogenously supplied during the priming first expansion is at or about 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, 2:1, 2.1:1, 2.2:1, 2.3:1, 2.4:1, 2.5:1, 2.6:1, 2.7:1, 2.8:1, 2.9:1, 3:1, 3.1:1, 3.2:1, 3.3:1, 3.4:1, 3.5:1, 3.6:1, 3.7:1, 3.8:1, 3.9:1, 4:1, 4.1:1, 4.2:1, 4.3:1, 4.4:1, 4.5:1, 4.6:1, 4.7:1, 4.8:1, 4.9:1, or 5:1.

[00928] In another embodiment, the number of APCs exogenously supplied during the priming first expansion is at or about 1×10^8, 1.1×10^8, 1.2×10^8, 1.3×10^8, 1.4×10^8, 1.5×10^8, 1.6×10^8, 1.7×10^8, 1.8×10^8, 1.9×10^8, 2×10^8, 2.1×10^8, 2.2×10^8, 2.3×10^8, 2.4×10^8, 2.5×10^8,

2.6×10^8, 2.7×10^8, 2.8×10^8, 2.9×10^8, 3×10^8, 3.1×10^8, 3.2×10^8, 3.3×10^8, 3.4×10^8 or 3.5×10^8 APCs, and the number of APCs exogenously supplied during the rapid second expansion is at or about 3.5×10^8, 3.6×10^8, 3.7×10^8, 3.8×10^8, 3.9×10^8, 4×10^8, 4.1×10^8, 4.2×10^8, 4.3×10^8, 4.4×10^8, 4.5×10^8, 4.6×10^8, 4.7×10^8, 4.8×10^8, 4.9×10^8, 5×10^8, 5.1×10^8, 5.2×10^8, 5.3×10^8, 5.4×10^8, 5.5×10^8, 5.6×10^8, 5.7×10^8, 5.8×10^8, 5.9×10^8, 6×10^8, 6.1×10^8, 6.2×10^8, 6.3×10^8, 6.4×10^8, 6.5×10^8, 6.6×10^8, 6.7×10^8, 6.8×10^8, 6.9×10^8, 7×10^8, 7.1×10^8, 7.2×10^8, 7.3×10^8, 7.4×10^8, 7.5×10^8, 7.6×10^8, 7.7×10^8, 7.8×10^8, 7.9×10^8, 8×10^8, 8.1×10^8, 8.2×10^8, 8.3×10^8, 8.4×10^8, 8.5×10^8, 8.6×10^8, 8.7×10^8, 8.8×10^8, 8.9×10^8, 9×10^8, 9.1×10^8, 9.2×10^8, 9.3×10^8, 9.4×10^8, 9.5×10^8, 9.6×10^8, 9.7×10^8, 9.8×10^8, 9.9×10^8 or 1×10^9 APCs.

[00929] In another embodiment, the number of APCs exogenously supplied during the priming first expansion is selected from the range of at or about 1.5×10^8 APCs to at or about 3×10^8 APCs, and the number of APCs exogenously supplied during the rapid second expansion is selected from the range of at or about 4×10^8 APCs to at or about 7.5×10^8 APCs.

[00930] In another embodiment, the number of APCs exogenously supplied during the priming first expansion is selected from the range of at or about 2×10^8 APCs to at or about 2.5×10^8 APCs, and the number of APCs exogenously supplied during the rapid second expansion is selected from the range of at or about 4.5×10^8 APCs to at or about 5.5×10^8 APCs.

[00931] In another embodiment, the number of APCs exogenously supplied during the priming first expansion is at or about 2.5×10^8 APCs, and the number of APCs exogenously supplied during the rapid second expansion is at or about 5×10^8 APCs.

[00932] In an embodiment, the number of APCs (including, for example, PBMCs) added at day 0 of the priming first expansion is approximately one-half of the number of PBMCs added at day 7 of the priming first expansion (e.g., day 7 of the method). In certain embodiments, the method comprises adding antigen presenting cells at day 0 of the priming first expansion to the first population of TILs and adding antigen presenting cells at day 7 to the second population of TILs, wherein the number of antigen presenting cells added at day 0 is approximately 50% of the number of antigen presenting cells added at day 7 of the priming first expansion (e.g., day 7 of the method).

[00933] In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is greater than the number of PBMCs exogenously supplied at day 0 of the priming first expansion.

[00934] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about 1.0×10^6 APCs/cm^2 to at or about 4.5×10^6 APCs/cm^2.

[00935] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about 1.5×10^6 APCs/cm^2 to at or about 3.5×10^6 APCs/cm^2.

[00936] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about 2×10^6 APCs/cm^2 to at or about 3×10^6 APCs/cm^2.

[00937] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density of at or about 2×10^6 APCs/cm^2.

[00938] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density of at or about 1.0×10^6, 1.1×10^6, 1.2×10^6, 1.3×10^6, 1.4×10^6, 1.5×10^6, 1.6×10^6, 1.7×10^6, 1.8×10^6, 1.9×10^6, 2×10^6, 2.1×10^6, 2.2×10^6, 2.3×10^6, 2.4×10^6, 2.5×10^6, 2.6×10^6, 2.7×10^6, 2.8×10^6, 2.9×10^6, 3×10^6, 3.1×10^6, 3.2×10^6, 3.3×10^6, 3.4×10^6, 3.5×10^6, 3.6×10^6, 3.7×10^6, 3.8×10^6, 3.9×10^6, 4×10^6, 4.1×10^6, 4.2×10^6, 4.3×10^6, 4.4×10^6 or 4.5×10^6 APCs/cm^2.

[00939] In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 2.5×10^6 APCs/cm^2 to at or about 7.5×10^6 APCs/cm^2.

[00940] In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 3.5×10^6 APCs/cm^2 to about 6.0×10^6 APCs/cm^2.

[00941] In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 4.0×10^6 APCs/cm^2 to about 5.5×10^6 APCs/cm^2.

[00942] In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 4.0×10^6 APCs/cm^2.

[00943] In another embodiment, the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density of at or about 2.5×10^6 APCs/cm^2,

2.6×10^6 APCs/cm^2, 2.7×10^6 APCs/cm^2, 2.8×10^6, 2.9×10^6, 3×10^6, 3.1×10^6, 3.2×10^6, 3.3×10^6, 3.4×10^6, 3.5×10^6, 3.6×10^6, 3.7×10^6, 3.8×10^6, 3.9×10^6, 4×10^6, 4.1×10^6, 4.2×10^6, 4.3×10^6, 4.4×10^6, 4.5×10^6, 4.6×10^6, 4.7×10^6, 4.8×10^6, 4.9×10^6, 5×10^6, 5.1×10^6, 5.2×10^6, 5.3×10^6, 5.4×10^6, 5.5×10^6, 5.6×10^6, 5.7×10^6, 5.8×10^6, 5.9×10^6, 6×10^6, 6.1×10^6, 6.2×10^6, 6.3×10^6, 6.4×10^6, 6.5×10^6, 6.6×10^6, 6.7×10^6, 6.8×10^6, 6.9×10^6, 7×10^6, 7.1×10^6, 7.2×10^6, 7.3×10^6, 7.4×10^6 or 7.5×10^6 APCs/cm^2.

[00944] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density of at or about 1.0×10^6, 1.1×10^6, 1.2×10^6, 1.3×10^6, 1.4×10^6, 1.5×10^6, 1.6×10^6, 1.7×10^6, 1.8×10^6, 1.9×10^6, 2×10^6, 2.1×10^6, 2.2×10^6, 2.3×10^6, 2.4×10^6, 2.5×10^6, 2.6×10^6, 2.7×10^6, 2.8×10^6, 2.9×10^6, 3×10^6, 3.1×10^6, 3.2×10^6, 3.3×10^6, 3.4×10^6, 3.5×10^6, 3.6×10^6, 3.7×10^6, 3.8×10^6, 3.9×10^6, 4×10^6, 4.1×10^6, 4.2×10^6, 4.3×10^6, 4.4×10^6 or 4.5×10^6 APCs/cm^2 and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density of at or about 2.5×10^6 APCs/cm^2, 2.6×10^6 APCs/cm^2, 2.7×10^6 APCs/cm^2, 2.8×10^6, 2.9×10^6, 3×10^6, 3.1×10^6, 3.2×10^6, 3.3×10^6, 3.4×10^6, 3.5×10^6, 3.6×10^6, 3.7×10^6, 3.8×10^6, 3.9×10^6, 4×10^6, 4.1×10^6, 4.2×10^6, 4.3×10^6, 4.4×10^6, 4.5×10^6, 4.6×10^6, 4.7×10^6, 4.8×10^6, 4.9×10^6, 5×10^6, 5.1×10^6, 5.2×10^6, 5.3×10^6, 5.4×10^6, 5.5×10^6, 5.6×10^6, 5.7×10^6, 5.8×10^6, 5.9×10^6, 6×10^6, 6.1×10^6, 6.2×10^6, 6.3×10^6, 6.4×10^6, 6.5×10^6, 6.6×10^6, 6.7×10^6, 6.8×10^6, 6.9×10^6, 7×10^6, 7.1×10^6, 7.2×10^6, 7.3×10^6, 7.4×10^6 or 7.5×10^6 APCs/cm^2.

[00945] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about 1.0×10^6 APCs/cm^2 to at or about 4.5×10^6 APCs/cm^2, and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 2.5×10^6 APCs/cm^2 to at or about 7.5×10^6 APCs/cm^2.

[00946] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about 1.5×10^6 APCs/cm^2 to at or about 3.5×10^6 APCs/cm^2, and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 3.5×10^6 APCs/cm^2 to at or about 6×10^6 APCs/cm^2.

[00947] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density selected from a range of at or about 2×10^6 APCs/cm^2 to at or about 3×10^6 APCs/cm^2, and the APCs exogenously supplied in the

rapid second expansion are seeded in the culture flask at a density selected from a range of at or about 4×10^6 APCs/cm^2 to at or about 5.5×10^6 APCs/cm^2.

[00948] In another embodiment, the APCs exogenously supplied in the priming first expansion are seeded in the culture flask at a density at or about 2×10^6 APCs/cm^2 and the APCs exogenously supplied in the rapid second expansion are seeded in the culture flask at a density of at or about 4×10^6 APCs/cm^2.

[00949] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of PBMCs exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 20:1.

[00950] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of PBMCs exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 10:1.

[00951] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of PBMCs exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 9:1.

[00952] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 8:1.

[00953] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 7:1.

[00954] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 6:1.

[00955] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 5:1.

[00956] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 4:1.

[00957] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 3:1.

[00958] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.9:1.

[00959] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.8:1.

[00960] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.7:1.

[00961] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.6:1.

[00962] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.5:1.

[00963] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.4:1.

[00964] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.3:1.

[00965] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.2:1.

[00966] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2.1:1.

[00967] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 1.1:1 to at or about 2:1.

[00968] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 10:1.

[00969] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 5:1.

[00970] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 4:1.

[00971] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 3:1.

[00972] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.9:1.

[00973] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.8:1.

[00974] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.7:1.

[00975] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.6:1.

[00976] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.5:1.

[00977] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.4:1.

[00978] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.3:1.

[00979] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about about 2:1 to at or about 2.2:1.

[00980] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from a range of from at or about 2:1 to at or about 2.1:1.

[00981] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about 2:1.

[00982] In another embodiment, the ratio of the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion to the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, 1.9:1, 2:1, 2.1:1, 2.2:1, 2.3:1, 2.4:1, 2.5:1, 2.6:1, 2.7:1, 2.8:1, 2.9:1, 3:1, 3.1:1, 3.2:1, 3.3:1, 3.4:1, 3.5:1, 3.6:1, 3.7:1, 3.8:1, 3.9:1, 4:1, 4.1:1, 4.2:1, 4.3:1, 4.4:1, 4.5:1, 4.6:1, 4.7:1, 4.8:1, 4.9:1, or 5:1.

[00983] In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about 1×10^8, 1.1×10^8, 1.2×10^8, 1.3×10^8, 1.4×10^8, 1.5×10^8, 1.6×10^8, 1.7×10^8, 1.8×10^8, 1.9×10^8, 2×10^8, 2.1×10^8, 2.2×10^8, 2.3×10^8, 2.4×10^8, 2.5×10^8, 2.6×10^8, 2.7×10^8, 2.8×10^8, 2.9×10^8, 3×10^8, 3.1×10^8, 3.2×10^8, 3.3×10^8, 3.4×10^8 or 3.5×10^8 APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is at or about 3.5×10^8, 3.6×10^8, 3.7×10^8, 3.8×10^8, 3.9×10^8, 4×10^8, 4.1×10^8, 4.2×10^8, 4.3×10^8, 4.4×10^8, 4.5×10^8, 4.6×10^8, 4.7×10^8, 4.8×10^8, 4.9×10^8, 5×10^8, 5.1×10^8, 5.2×10^8, 5.3×10^8, 5.4×10^8, 5.5×10^8, 5.6×10^8, 5.7×10^8, 5.8×10^8, 5.9×10^8, 6×10^8, 6.1×10^8, 6.2×10^8, 6.3×10^8, 6.4×10^8, 6.5×10^8, 6.6×10^8, 6.7×10^8, 6.8×10^8, 6.9×10^8, 7×10^8, 7.1×10^8, 7.2×10^8, 7.3×10^8, 7.4×10^8, 7.5×10^8, 7.6×10^8, 7.7×10^8, 7.8×10^8, 7.9×10^8, 8×10^8, 8.1×10^8, 8.2×10^8, 8.3×10^8, 8.4×10^8, 8.5×10^8, 8.6×10^8, 8.7×10^8, 8.8×10^8, 8.9×10^8, 9×10^8, 9.1×10^8, 9.2×10^8, 9.3×10^8, 9.4×10^8, 9.5×10^8, 9.6×10^8, 9.7×10^8, 9.8×10^8, 9.9×10^8 or $1x10^9$ APCs (including, for example, PBMCs).

[00984] In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from the range of at or about 1×10^8 APCs (including, for example, PBMCs) to at or about 3.5×10^8 APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is selected from the range of at or about 3.5×10^8 APCs (including, for example, PBMCs) to at or about 1×10^9 APCs (including, for example, PBMCs).

[00985] In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from the range of at or about 1.5×10^8 APCs to at or about 3×10^8 APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is selected from the range of at or about 4×10^8 APCs (including, for example, PBMCs) to at or about 7.5×10^8 APCs (including, for example, PBMCs).

[00986] In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is selected from the range of at or about 2×10^8 APCs (including, for example, PBMCs) to at or about 2.5×10^8 APCs (including, for example, PBMCs), and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is selected from the range of at or about 4.5×10^8 APCs (including, for example, PBMCs) to at or about 5.5×10^8 APCs (including, for example, PBMCs).

[00987] In another embodiment, the number of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion is at or about 2.5×10^8 APCs (including, for example, PBMCs) and the number of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is at or about 5×10^8 APCs (including, for example, PBMCs)

[00988] In an embodiment, the number of layers of APCs (including, for example, PBMCs) added at day 0 of the priming first expansion is approximately one-half of the number of layers of APCs (including, for example, PBMCs) added at day 7 of the rapid second expansion. In certain embodiments, the method comprises adding antigen presenting cell layers at day 0 of the priming first expansion to the first population of TILs and adding antigen presenting cell layers at day 7 to the second population of TILs, wherein the number

of antigen presenting cell layer added at day 0 is approximately 50% of the number of antigen presenting cell layers added at day 7.

[00989] In another embodiment, the number of layers of APCs (including, for example, PBMCs) exogenously supplied at day 7 of the rapid second expansion is greater than the number of layers of APCs (including, for example, PBMCs) exogenously supplied at day 0 of the priming first expansion.

[00990] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 4 cell layers.

[00991] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about one cell layer and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3 cell layers.

[00992] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 1.5 cell layers to at or about 2.5 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3 cell layers.

[00993] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about one cell layer and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers.

[00994] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of of at or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9 or 8 cell layers.

[00995] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 1 cell layer to at or about 2 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3 cell layers to at or about 10 cell layers.

[00996] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers to at or about 3 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 4 cell layers to at or about 8 cell layers.

[00997] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 2 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 4 cell layers to at or about 8 cell layers.

[00998] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 1, 2 or 3 cell layers and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with an average thickness of at or about 3, 4, 5, 6, 7, 8, 9 or 10 cell layers.

[00999] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:10.

[001000] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:8.

[001001] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:7.

[001002] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:6.

[001003] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:5.

[001004] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:4.

[001005] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:3.

[001006] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.1 to at or about 1:2.

[001007] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.2 to at or about 1:8.

[001008] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.3 to at or about 1:7.

[001009] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.4 to at or about 1:6.

[001010] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.5 to at or about 1:5.

[001011] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.6 to at or about 1:4.

[001012] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.7 to at or about 1:3.5.

[001013] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.8 to at or about 1:3.

[001014] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from the range of at or about 1:1.9 to at or about 1:2.5.

[001015] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is at or about 1: 2.

[001016] In another embodiment, day 0 of the priming first expansion occurs in the presence of layered APCs (including, for example, PBMCs) with a first average thickness equal to a first number of layers of APCs (including, for example, PBMCs) and day 7 of the rapid second expansion occurs in the presence of layered APCs (including, for example, PBMCs)

with a second average thickness equal to a second number of layers of APCs (including, for example, PBMCs), wherein the ratio of the first number of layers of APCs (including, for example, PBMCs) to the second number of layers of APCs (including, for example, PBMCs) is selected from at or about 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9, 1:2, 1:2.1, 1:2.2, 1:2.3, 1:2.4, 1:2.5, 1:2.6, 1:2.7, 1:2.8, 1:2.9, 1:3, 1:3.1, 1:3.2, 1:3.3, 1:3.4, 1:3.5, 1:3.6, 1:3.7, 1:3.8, 1:3.9, 1:4, 1:4.1, 1:4.2, 1:4.3, 1:4.4, 1:4.5, 1:4.6, 1:4.7, 1:4.8, 1:4.9, 1:5, 1:5.1, 1:5.2, 1:5.3, 1:5.4, 1:5.5, 1:5.6, 1:5.7, 1:5.8, 1:5.9, 1:6, 1:6.1, 1:6.2, 1:6.3, 1:6.4, 1:6.5, 1:6.6, 1:6.7, 1:6.8, 1:6.9, 1:7, 1:7.1, 1:7.2, 1:7.3, 1:7.4, 1:7.5, 1:7.6, 1:7.7, 1:7.8, 1:7.9, 1:8, 1:8.1, 1:8.2, 1:8.3, 1:8.4, 1:8.5, 1:8.6, 1:8.7, 1:8.8, 1:8.9, 1:9, 1:9.1, 1:9.2, 1:9.3, 1:9.4, 1:9.5, 1:9.6, 1:9.7, 1:9.8, 1:9.9 or 1:10.

[001017] In some embodiments, the number of APCs in the priming first expansion is selected from the range of about 1.0×10^6 APCs/cm^2 to about 4.5×10^6 APCs/cm^2, and the number of APCs in the rapid second expansion is selected from the range of about 2.5×10^6 APCs/cm^2 to about 7.5×10^6 APCs/cm^2.

[001018] In some embodiments, the number of APCs in the priming first expansion is selected from the range of about 1.5×10^6 APCs/cm^2 to about 3.5×10^6 APCs/cm^2, and the number of APCs in the rapid second expansion is selected from the range of about 3.5×10^6 APCs/cm^2 to about 6.0×10^6 APCs/cm^2.

[001019] In some embodiments, the number of APCs in the priming first expansion is selected from the range of about 2.0×10^6 APCs/cm^2 to about 3.0×10^6 APCs/cm^2, and the number of APCs in the rapid second expansion is selected from the range of about 4.0×10^6 APCs/cm^2 to about 5.5×10^6 APCs/cm^2.

H. Optional Cell Medium Components

1. Anti-CD3 Antibodies

[001020] In some embodiments, the culture media used in expansion methods described herein (see for example, Figure 8 (in particular, *e.g.*, Figure 8B)) include an anti-CD3 antibody. An anti-CD3 antibody in combination with IL-2 induces T cell activation and cell division in the TIL population. This effect can be seen with full length antibodies as well as

Fab and F(ab')2 fragments, with the former being generally preferred; see, *e.g.,* Tsoukas *et al.*, *J. Immunol.* **1985,** *135,* 1719, hereby incorporated by reference in its entirety.

[001021] As will be appreciated by those in the art, there are a number of suitable anti-human CD3 antibodies that find use in the invention, including anti-human CD3 polyclonal and monoclonal antibodies from various mammals, including, but not limited to, murine, human, primate, rat, and canine antibodies. In particular embodiments, the OKT3 anti-CD3 antibody is used (commercially available from Ortho-McNeil, Raritan, NJ or Miltenyi Biotech, Auburn, CA).

TABLE 21: 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 NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY DDHYCLDYWG QGTTLTVSSA KTTAPSVYPL APVCGGTTGS SVTLGCLVKG YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL YTLSSSVTVT SSTWPSQSIT CNVAHPASST KVDKKIEPRP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSRDE LTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:2 Muromonab light chain	QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT SKLASGVPAH FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG TKLEINRADT APTVSIFPPS SEQLTSGGAS VVCFLNNFYP KDINVKWKID GSERQNGVLN SWTDQDSKDS TYSMSSTLTL TKDEYERHNS YTCEATHKTS TSPIVKSFNR NEC	60 120 180 213

2. 4-1BB (CD137) AGONISTS

[001022] In an embodiment, the cell culture medium of the priming first expansion and/or the rapid second expansion comprises a TNFRSF agonist. In an embodiment, the TNFRSF agonist is a 4-1BB (CD137) agonist. The 4-1BB agonist may be any 4-1BB binding molecule known in the art. The 4-1BB binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian 4-1BB. The 4-1BB agonists or 4-1BB binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The 4-1BB agonist or 4-1BB binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that

bind to 4-1BB. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the 4-1BB agonist is an antigen binding protein that is a humanized antibody. In some embodiments, 4-1BB agonists for use in the presently disclosed methods and compositions include anti-4-1BB antibodies, human anti-4-1BB antibodies, mouse anti-4-1BB antibodies, mammalian anti-4-1BB antibodies, monoclonal anti-4-1BB antibodies, polyclonal anti-4-1BB antibodies, chimeric anti-4-1BB antibodies, anti-4-1BB adnectins, anti-4-1BB domain antibodies, single chain anti-4-1BB fragments, heavy chain anti-4-1BB fragments, light chain anti-4-1BB fragments, anti-4-1BB fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. Agonistic anti-4-1BB antibodies are known to induce strong immune responses. Lee, *et al.*, *PLOS One* **2013**, *8,* e69677. In a preferred embodiment, the 4-1BB agonist is an agonistic, anti-4-1BB humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line). In an embodiment, the 4-1BB agonist is EU-101 (Eutilex Co. Ltd.), utomilumab, or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof. In a preferred embodiment, the 4-1BB agonist is utomilumab or urelumab, or a fragment, derivative, conjugate, variant, or biosimilar thereof.

[001023] In a preferred embodiment, the 4-1BB agonist or 4-1BB binding molecule may also be a fusion protein. In a preferred embodiment, a multimeric 4-1BB agonist, such as a trimeric or hexameric 4-1BB agonist (with three or six ligand binding domains), may induce superior receptor (4-1BBL) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

[001024] Agonistic 4-1BB antibodies and fusion proteins are known to induce strong immune responses. In a preferred embodiment, the 4-1BB agonist is a monoclonal antibody or fusion protein that binds specifically to 4-1BB antigen in a manner sufficient to reduce toxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion

protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the 4-1BB agonist is an agonistic 4-1BB monoclonal antibody or fusion protein which abrogates Fc region functionality.

[001025] In some embodiments, the 4-1BB agonists are characterized by binding to human 4-1BB (SEQ ID NO:9) with high affinity and agonistic activity. In an embodiment, the 4-1BB agonist is a binding molecule that binds to human 4-1BB (SEQ ID NO:9). In an embodiment, the 4-1BB agonist is a binding molecule that binds to murine 4-1BB (SEQ ID NO:10). The amino acid sequences of 4-1BB antigen to which a 4-1BB agonist or binding molecule binds are summarized in Table 22.

TABLE 22. Amino acid sequences of 4-1BB antigens.

Identifier	Sequence (One-Letter Amino Acid Symbols)
SEQ ID NO:9 human 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Homo sapiens)	MGNSCYNIVA TLLLVLNFER TRSLQDPCSN CPAGTFCDNN RNQICSPCPP NSFSSAGGQR 60 TCDICRQCKG VFRTRKECSS TSNAECDCTP GFHCLGAGCS MCEQDCKQGQ ELTKKGCKDC 120 CFGTFNDQKR GICRPWTNCS LDGKSVLVNG TKERDVVCGP SPADLSPGAS SVTPPAPARE 180 PGHSPQIISF FLALTSTALL FLLFFLTLRF SVVKRGRKKL LYIFKQPFMR PVQTTQEEDG 240 CSCRFPEEEE GGCEL 255
SEQ ID NO:10 murine 4-1BB, Tumor necrosis factor receptor superfamily, member 9 (Mus musculus)	MGNNCYNVVV IVLLLVGCEK VGAVQNSCDN CQPGTFCRKY NPVCKSCPPS TFSSIGGQPN 60 CNICRVCAGY FRFKKFCSST HNAECECIEG FHCLGPQCTR CEKDCRPGQE LTKQGCKTCS 120 LGTFNDQNGT GVCRPWTNCS LDGRSVLKTG TTEKDVVCGP PVVSFSPSTT ISVTPEGGPG 180 GHSLQVLTLF LALTSALLLA LIFITLLFSV LKWIRKKFPH IFKQPFKKTT GAAQEEDACS 240 CRCPQEEEGG GGGYEL 256

[001026] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds human or murine 4-1BB with a K_D of about 100 pM or lower, binds human or murine 4-1BB with a K_D of about 90 pM or lower, binds human or murine 4-1BB with a K_D of about 80 pM or lower, binds human or murine 4-1BB with a K_D of about 70 pM or lower, binds human or murine 4-1BB with a K_D of about 60 pM or lower, binds human or murine 4-1BB with a K_D of about 50 pM or lower, binds human or murine 4-1BB with a K_D of about 40 pM or lower, or binds human or murine 4-1BB with a K_D of about 30 pM or lower.

[001027] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human or murine 4-1BB with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human or murine 4-1BB with a

k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human or murine 4-1BB with a k_{assoc} of about 1×10^6 1/M·s or faster.

[001028] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with a k_{dissoc} of about 2×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower , binds to human or murine 4-1BB with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human or murine 4-1BB with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds to human or murine 4-1BB with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human or murine 4-1BB with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[001029] In some embodiments, the compositions, processes and methods described include a 4-1BB agonist that binds to human or murine 4-1BB with an IC_{50} of about 10 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 9 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 8 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 7 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 6 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 5 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 4 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 3 nM or lower, binds to human or murine 4-1BB with an IC_{50} of about 2 nM or lower, or binds to human or murine 4-1BB with an IC_{50} of about 1 nM or lower.

[001030] In a preferred embodiment, the 4-1BB agonist is utomilumab, also known as PF-05082566 or MOR-7480, or a fragment, derivative, variant, or biosimilar thereof. Utomilumab is available from Pfizer, Inc. Utomilumab is an immunoglobulin G2-lambda, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor (TNFR) superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of utomilumab are set forth in Table 7. Utomilumab comprises glycosylation sites at Asn59 and Asn292; heavy chain intrachain disulfide bridges at positions 22-96 (V_H-V_L), 143-199 (C_H1-C_L), 256-316 (C_H2) and 362-420 (C_H3); light chain intrachain disulfide bridges at positions 22'-87' (V_H-V_L) and 136'-195' (C_H1-C_L); interchain heavy chain-heavy chain disulfide bridges at IgG2A isoform positions 218-218, 219-219, 222-222, and 225-225, at IgG2A/B isoform positions 218-130, 219-219, 222-222, and 225-

225, and at IgG2B isoform positions 219-130 (2), 222-222, and 225-225; and interchain heavy chain-light chain disulfide bridges at IgG2A isoform positions 130-213' (2), IgG2A/B isoform positions 218-213' and 130-213', and at IgG2B isoform positions 218-213' (2). The preparation and properties of utomilumab and its variants and fragments are described in U.S. Patent Nos. 8,821,867; 8,337,850; and 9,468,678, and International Patent Application Publication No. WO 2012/032433 A1, the disclosures of each of which are incorporated by reference herein. Preclinical characteristics of utomilumab are described in Fisher, *et al.*, *Cancer Immunolog. & Immunother.* **2012,** *61,* 1721-33. Current clinical trials of utomilumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02444793, NCT01307267, NCT02315066, and NCT02554812.

[001031] In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:11 and a light chain given by SEQ ID NO:12. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:11 and SEQ ID NO:12, respectively.

[001032] In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of utomilumab. In an embodiment, the 4-1BB agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:13, and the 4-1BB agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:14, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H

and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14.

[001033] In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:15, SEQ ID NO:16, and SEQ ID NO:17, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:18, SEQ ID NO:19, and SEQ ID NO:20, respectively, and conservative amino acid substitutions thereof.

[001034] In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to utomilumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is utomilumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological

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

TABLE 23. Amino acid sequences for 4-1BB agonist antibodies related to utomilumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:11 heavy chain for utomilumab	EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMGK IYPGDSYTNY SPSFQGQVTI SADKSISTAY LQWSSLKASD TAMYYCARGY GIFDYWGQGT LVTVSSASTK GPSVFPLAPC SRSTSESTAA LGCLVKDYFP EPVTVSWNSG ALTSGVHTFP AVLQSSGLYS LSSVVTVPSS NFGTQTYTCN VDHKPSNTKV DKTVERKCCV ECPPCPAPPV AGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVQF NWYVDGVEVH NAKTKPREEQ FNSTFRVVSV LTVVHQDWLN GKEYKCKVSN KGLPAPIEKT ISKTKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP PMLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP G	60 120 180 240 300 360 420 441
SEQ ID NO:12 light chain for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVLGQ PKAAPSVTLF PPSSEELQAN KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTPSKQS NNKYAASSYL SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS	60 120 180 214
SEQ ID NO:13 heavy chain variable region for utomilumab	EVQLVQSGAE VKKPGESLRI SCKGSGYSFS TYWISWVRQM PGKGLEWMG KIYPGDSYTN YSPSFQGQVT ISADKSISTA YLQWSSLKAS DTAMYYCARG YGIFDYWGQ GTLVTVSS	60 118
SEQ ID NO:14 light chain variable region for utomilumab	SYELTQPPSV SVSPGQTASI TCSGDNIGDQ YAHWYQQKPG QSPVLVIYQD KNRPSGIPER FSGSNSGNTA TLTISGTQAM DEADYYCATY TGFGSLAVFG GGTKLTVL	60 108
SEQ ID NO:15 heavy chain CDR1 for utomilumab	STYWIS	6
SEQ ID NO:16 heavy chain CDR2 for utomilumab	KIYPGDSYTN YSPSFQG	17
SEQ ID NO:17 heavy chain CDR3 for utomilumab	RGYGIFDY	8
SEQ ID NO:18 light chain CDR1 for utomilumab	SGDNIGDQYA H	11
SEQ ID NO:19 light chain CDR2 for utomilumab	QDKNRPS	7
SEQ ID NO:20 light chain CDR3 for utomilumab	ATYTGFGSLA V	11

[001035] In a preferred embodiment, the 4-1BB agonist is the monoclonal antibody urelumab, also known as BMS-663513 and 20H4.9.h4a, or a fragment, derivative, variant, or biosimilar thereof. Urelumab is available from Bristol-Myers Squibb, Inc., and Creative Biolabs, Inc. Urelumab is an immunoglobulin G4-kappa, anti-[*Homo sapiens* TNFRSF9 (tumor necrosis factor receptor superfamily member 9, 4-1BB, T cell antigen ILA, CD137)], *Homo sapiens* (fully human) monoclonal antibody. The amino acid sequences of urelumab are set forth in Table EE. Urelumab comprises N-glycosylation sites at positions 298 (and 298'); heavy chain intrachain disulfide bridges at positions 22-95 (V_H-V_L), 148-204 (C_H1-C_L), 262-322

(C_H2) and 368-426 (C_H3) (and at positions 22''-95'', 148''-204'', 262''-322'', and 368''-426''); light chain intrachain disulfide bridges at positions 23'-88' (V_H-V_L) and 136'-196' (C_H1-C_L) (and at positions 23'''-88''' and 136'''-196'''); interchain heavy chain-heavy chain disulfide bridges at positions 227-227'' and 230-230''; and interchain heavy chain-light chain disulfide bridges at 135-216' and 135''-216'''. The preparation and properties of urelumab and its variants and fragments are described in U.S. Patent Nos. 7,288,638 and 8,962,804, the disclosures of which are incorporated by reference herein. The preclinical and clinical characteristics of urelumab are described in Segal, *et al.*, *Clin. Cancer Res.* **2016,** *available at* http:/dx.doi.org/ 10.1158/1078-0432.CCR-16-1272. Current clinical trials of urelumab in a variety of hematological and solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT01775631, NCT02110082, NCT02253992, and NCT01471210.

[001036] In an embodiment, a 4-1BB agonist comprises a heavy chain given by SEQ ID NO:21 and a light chain given by SEQ ID NO:22. In an embodiment, a 4-1BB agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively. In an embodiment, a 4-1BB agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:21 and SEQ ID NO:22, respectively.

[001037] In an embodiment, the 4-1BB agonist comprises the heavy and light chain CDRs or variable regions (VRs) of urelumab. In an embodiment, the 4-1BB agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:23, and the 4-1BB agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:24, and conservative amino acid substitutions thereof. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID

NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively. In an embodiment, a 4-1BB agonist comprises an scFv antibody comprising V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24.

[001038] In an embodiment, a 4-1BB agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:25, SEQ ID NO:26, and SEQ ID NO:27, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30, respectively, and conservative amino acid substitutions thereof.

[001039] In an embodiment, the 4-1BB agonist is a 4-1BB agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to urelumab. In an embodiment, the biosimilar monoclonal antibody comprises an 4-1BB antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a 4-1BB agonist antibody authorized or submitted for authorization, wherein the 4-1BB agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is urelumab. The 4-1BB agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or

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

TABLE 24: Amino acid sequences for 4-1BB agonist antibodies related to urelumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:21 heavy chain for urelumab	QVQLQQWGAG LLKPSETLSL TCAVYGGSFS GYYWSWIRQS PEKGLEWIGE INHGGYVTYN PSLESRVTIS VDTSKNQFSL KLSSVTAADT AVYYCARDYG PGNYDWYFDL WGRGTLVTVS SASTKGPSVF PLAPCSRSTS ESTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTK TYTCNVDHKP SNTKVDKRVE SKYGPPCPPC PAPEFLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSQE DPEVQFNWYV DGVEVHNAKT KPREEQFNST YRVVSVLTVL HQDWLNGKEY KCKVSNKGLP SSIEKTISKA KGQPREPQVY TLPPSQEEMT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD SDGSFFLYSR LTVDKSRWQE GNVFSCSVMH EALHNHYTQK SLSLSLGK	60 120 180 240 300 360 420 448
SEQ ID NO:22 light chain for urelumab	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPALTF CGGTKVEIKR TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYSLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC	60 120 180 216
SEQ ID NO:23 variable heavy chain for urelumab	MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YYWSWIRQSP EKGLEWIGEI NHGGYVTYNP SLESRVTISV DTSKNQFSLK LSSVTAADTA VYYCARDYGP	60 120
SEQ ID NO:24 variable light chain for urelumab	MEAPAQLLFL LLLWLPDTTG EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ	60 110
SEQ ID NO:25 heavy chain CDR1 for urelumab	GYYWS	5
SEQ ID NO:26 heavy chain CDR2 for urelumab	EINHGGYVTY NPSLES	16
SEQ ID NO:27 heavy chain CDR3 for urelumab	DYGPGNYDWY FDL	13
SEQ ID NO:28 light chain CDR1 for urelumab	RASQSVSSYL A	11
SEQ ID NO:29 light chain CDR2 for urelumab	DASNRAT	7
SEQ ID NO:30 light chain CDR3 for urelumab	QQRSDWPPAL T	11

[001040] In an embodiment, the 4-1BB agonist is selected from the group consisting of 1D8, 3Elor, 4B4 (BioLegend 309809), H4-1BB-M127 (BD Pharmingen 552532), BBK2 (Thermo Fisher MS621PABX), 145501 (Leinco Technologies B591), the antibody produced by cell line deposited as ATCC No. HB-11248 and disclosed in U.S. Patent No. 6,974,863, 5F4 (BioLegend 31 1503), C65-485 (BD Pharmingen 559446), antibodies disclosed in U.S. Patent Application Publication No. US 2005/0095244, antibodies disclosed in U.S. Patent No. 7,288,638 (such as 20H4.9-IgGl (BMS-663031)), antibodies disclosed in U.S. Patent No. 6,887,673 (such as 4E9 or BMS-554271), antibodies disclosed in U.S. Patent No. 7,214,493,

antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in U.S. Patent No. 6,905,685 (such as 4E9 or BMS-554271), antibodies disclosed in U.S. Patent No. 6,362,325 (such as 1D8 or BMS-469492; 3H3 or BMS-469497; or 3El), antibodies disclosed in U.S. Patent No. 6,974,863 (such as 53A2); antibodies disclosed in U.S. Patent No. 6,210,669 (such as 1D8, 3B8, or 3El), antibodies described in U.S. Patent No. 5,928,893, antibodies disclosed in U.S. Patent No. 6,303,121, antibodies disclosed in U.S. Patent No. 6,569,997, antibodies disclosed in International Patent Application Publication Nos. WO 2012/177788, WO 2015/119923, and WO 2010/042433, and fragments, derivatives, conjugates, variants, or biosimilars thereof, wherein the disclosure of each of the foregoing patents or patent application publications is incorporated by reference here.

[001041] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein described in International Patent Application Publication Nos. WO 2008/025516 A1, WO 2009/007120 A1, WO 2010/003766 A1, WO 2010/010051 A1, and WO 2010/078966 A1; U.S. Patent Application Publication Nos. US 2011/0027218 A1, US 2015/0126709 A1, US 2011/0111494 A1, US 2015/0110734 A1, and US 2015/0126710 A1; and U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

[001042] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof (See, Figure 50). In structures I-A and I-B, the cylinders refer to individual polypeptide binding domains. Structures I-A and I-B comprise three linearly-linked TNFRSF binding domains derived from *e.g.*, 4-1BBL (4-1BB ligand, CD137 ligand (CD137L), or tumor necrosis factor superfamily member 9 (TNFSF9)) or an antibody that binds 4-1BB, which fold to form a trivalent protein, which is then linked to a second triavelent protein through IgG1-Fc (including C_H3 and C_H2 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. Any scFv domain design may

be used, such as those described in de Marco, *Microbial Cell Factories*, **2011,** *10*, 44; Ahmad, *et al.*, *Clin. & Dev. Immunol.* **2012,** 980250; Monnier, *et al.*, *Antibodies,* **2013,** *2,* 193-208; or in references incorporated elsewhere herein. Fusion protein structures of this form are described in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein.

[001043] Amino acid sequences for the other polypeptide domains of structure I-A are given in Table 9. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides.

TABLE 25: Amino acid sequences for TNFRSF agonist fusion proteins, including 4-1BB agonist fusion proteins, with C-terminal Fc-antibody fragment fusion protein design (structure I-A).

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:31 Fc domain	KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 230
SEQ ID NO:32 linker	GGPGSSKSCD KTHTCPPCPA PE	22
SEQ ID NO:33 linker	GGSGSSKSCD KTHTCPPCPA PE	22
SEQ ID NO:34 linker	GGPGSSSSSS SKSCDKTHTC PPCPAPE	27
SEQ ID NO:35 linker	GGSGSSSSSS SKSCDKTHTC PPCPAPE	27
SEQ ID NO:36 linker	GGPGSSSSSS SSSKSCDKTH TCPPCPAPE	29
SEQ ID NO:37 linker	GGSGSSSSSS SSSKSCDKTH TCPPCPAPE	29
SEQ ID NO:38 linker	GGPGSSGSGS SDKTHTCPPC PAPE	24
SEQ ID NO:39 linker	GGPGSSGSGS DKTHTCPPCP APE	23
SEQ ID NO:40 linker	GGPSSSGSDK THTCPPCPAP E	21
SEQ ID NO:41 linker	GGSSSSSSSS GSDKTHTCPP CPAPE	25

[001044] Amino acid sequences for the other polypeptide domains of structure I-B are given in Table 10. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

TABLE 26: Amino acid sequences for TNFRSF agonist fusion proteins, including 4-1BB agonist fusion proteins, with N-terminal Fc-antibody fragment fusion protein design (structure I-B).

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:42 Fc domain	METDTLLLWV LLLWVPAGNG DKTHTCPPCP APELLGGPSV FLFPPKPKDT LMISRTPEVT CVVVDVSHED PEVKFNWYVD GVEVHNAKTK PREEQYNSTY RVVSVLTVLH QDWLNGKEYK CKVSNKALPA PIEKTISKAK GQPREPQVYT LPPSREEMTK NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSKL TVDKSRWQQG NVFSCSVMHE ALHNHYTQKS LSLSPG	60 120 180 240 246
SEQ ID NO:43 linker	SGSGSGSGSG S	11
SEQ ID NO:44 linker	SSSSSSGSGS GS	12
SEQ ID NO:45 linker	SSSSSSGSGS GSGSGS	16

[001045] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains selected from the group consisting of a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain of urelumab, a variable heavy chain and variable light chain of utomilumab, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table 10, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[001046] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:46. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a soluble 4-1BBL sequence. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains comprising a sequence according to SEQ ID NO:47.

[001047] In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:13 and SEQ ID NO:14, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:23 and SEQ ID NO:24, respectively, wherein the V_H and V_L domains are connected by a linker. In an

embodiment, a 4-1BB agonist fusion protein according to structures I-A or I-B comprises one or more 4-1BB binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the V_H and V_L sequences given in Table 11, wherein the V_H and V_L domains are connected by a linker.

TABLE 27: Additional polypeptide domains useful as 4-1BB binding domains in fusion proteins or as scFv 4-1BB agonist antibodies.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:46 4-1BBL	MEYASDASLD PEAPWPPAPR ARACRVLPWA LVAGLLLLLL LAAACAVFLA CPWAVSGARA SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAALA LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ GATVLGLFRV TPEIPAGLPS PRSE	60 120 180 240 254
SEQ ID NO:47 4-1BBL soluble domain	LRQGMFAQLV AQNVLLIDGP LSWYSDPGLA GVSLTGGLSY KEDTKELVVA KAGVYYVFFQ LELRRVVAGE GSGSVSLALH LQPLRSAAGA AALALTVDLP PASSEARNSA FGFQGRLLHL SAGQRLGVHL HTEARARHAW QLTQGATVLG LFRVTPEIPA GLPSPRSE	60 120 168
SEQ ID NO:48 variable heavy chain for 4B4-1-1 version 1	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVS	60 118
SEQ ID NO:49 variable light chain for 4B4-1-1 version 1	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIK	60 107
SEQ ID NO:50 variable heavy chain for 4B4-1-1 version 2	QVQLQQPGAE LVKPGASVKL SCKASGYTFS SYWMHWVKQR PGQVLEWIGE INPGNGHTNY NEKFKSKATL TVDKSSSTAY MQLSSLTSED SAVYYCARSF TTARGFAYWG QGTLVTVSA	60 119
SEQ ID NO:51 variable light chain for 4B4-1-1 version 2	DIVMTQSPAT QSVTPGDRVS LSCRASQTIS DYLHWYQQKS HESPRLLIKY ASQSISGIPS RFSGSGSGSD FTLSINSVEP EDVGVYYCQD GHSFPPTFGG GTKLEIKR	60 108
SEQ ID NO:52 variable heavy chain for H39E3-2	MDWTWRILFL VAAATGAHSE VQLVESGGGL VQPGGSLRLS CAASGFTFSD YWMSWVRQAP GKGLEWVADI KNDGSYTNYA PSLTNRFTIS RDNAKNSLYL QMNSLRAEDT AVYYCARELT	60 120
SEQ ID NO:53 variable light chain for H39E3-2	MEAPAQLLFL LLLWLPDTTG DIVMTQSPDS LAVSLGERAT INCKSSQSLL SSGNQKNYL WYQQKPGQPP KLLIYYASTR QSGVPDRFSG SGSGTDFTLT ISSLQAEDVA	60 110

[001048] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble 4-1BB binding domain, (ii) a first peptide linker, (iii) a second soluble 4-1BB binding domain, (iv) a second peptide linker, and (v) a third soluble 4-1BB binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain, wherein each of the soluble 4-1BB domains lacks a stalk region (which contributes to trimerisation and provides a

certain distance to the cell membrane, but is not part of the 4-1BB binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

[001049] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain, (iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein each TNF superfamily cytokine domain is a 4-1BB binding domain.

[001050] In an embodiment, the 4-1BB agonist is a 4-1BB agonistic scFv antibody comprising any of the foregoing V_H domains linked to any of the foregoing V_L domains.

[001051] In an embodiment, the 4-1BB agonist is BPS Bioscience 4-1BB agonist antibody catalog no. 79097-2, commercially available from BPS Bioscience, San Diego, CA, USA. In an embodiment, the 4-1BB agonist is Creative Biolabs 4-1BB agonist antibody catalog no. MOM-18179, commercially available from Creative Biolabs, Shirley, NY, USA.

3. OX40 (CD134) AGONISTS

[001052] In an embodiment, the TNFRSF agonist is an OX40 (CD134) agonist. The OX40 agonist may be any OX40 binding molecule known in the art. The OX40 binding molecule may be a monoclonal antibody or fusion protein capable of binding to human or mammalian OX40. The OX40 agonists or OX40 binding molecules may comprise an immunoglobulin heavy chain of any isotype (*e.g.*, IgG, IgE, IgM, IgD, IgA, and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. The OX40 agonist or OX40 binding molecule may have both a heavy and a light chain. As used herein, the term binding molecule also includes antibodies (including full length antibodies), monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human, humanized or chimeric antibodies, and antibody fragments, *e.g.*, Fab fragments, F(ab′) fragments, fragments produced by a Fab expression library, epitope-binding fragments of any of the above, and engineered forms of antibodies, *e.g.*, scFv molecules, that bind to OX40. In an embodiment, the OX40 agonist is an antigen binding protein that is a fully human antibody. In an embodiment, the OX40 agonist is an antigen binding protein that is a humanized antibody. In some embodiments, OX40 agonists for use in the presently disclosed methods and compositions include anti-

OX40 antibodies, human anti-OX40 antibodies, mouse anti-OX40 antibodies, mammalian anti-OX40 antibodies, monoclonal anti-OX40 antibodies, polyclonal anti-OX40 antibodies, chimeric anti-OX40 antibodies, anti-OX40 adnectins, anti-OX40 domain antibodies, single chain anti-OX40 fragments, heavy chain anti-OX40 fragments, light chain anti-OX40 fragments, anti-OX40 fusion proteins, and fragments, derivatives, conjugates, variants, or biosimilars thereof. In a preferred embodiment, the OX40 agonist is an agonistic, anti-OX40 humanized or fully human monoclonal antibody (*i.e.*, an antibody derived from a single cell line).

[001053] In a preferred embodiment, the OX40 agonist or OX40 binding molecule may also be a fusion protein. OX40 fusion proteins comprising an Fc domain fused to OX40L are described, for example, in Sadun, *et al.*, *J. Immunother.* **2009,** *182,* 1481-89. In a preferred embodiment, a multimeric OX40 agonist, such as a trimeric or hexameric OX40 agonist (with three or six ligand binding domains), may induce superior receptor (OX40L) clustering and internal cellular signaling complex formation compared to an agonistic monoclonal antibody, which typically possesses two ligand binding domains. Trimeric (trivalent) or hexameric (or hexavalent) or greater fusion proteins comprising three TNFRSF binding domains and IgG1-Fc and optionally further linking two or more of these fusion proteins are described, *e.g.*, in Gieffers, *et al.*, *Mol. Cancer Therapeutics* **2013,** *12,* 2735-47.

[001054] Agonistic OX40 antibodies and fusion proteins are known to induce strong immune responses. Curti, *et al.*, *Cancer Res.* **2013**, *73,* 7189-98. In a preferred embodiment, the OX40 agonist is a monoclonal antibody or fusion protein that binds specifically to OX40 antigen in a manner sufficient to reduce toxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cellular toxicity (ADCC), for example NK cell cytotoxicity. In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates antibody-dependent cell phagocytosis (ADCP). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein that abrogates complement-dependent cytotoxicity (CDC). In some embodiments, the OX40 agonist is an agonistic OX40 monoclonal antibody or fusion protein which abrogates Fc region functionality.

[001055] In some embodiments, the OX40 agonists are characterized by binding to human OX40 (SEQ ID NO:54) with high affinity and agonistic activity. In an embodiment, the OX40 agonist is a binding molecule that binds to human OX40 (SEQ ID NO:54). In an embodiment, the OX40 agonist is a binding molecule that binds to murine OX40 (SEQ ID

NO:55). The amino acid sequences of OX40 antigen to which an OX40 agonist or binding molecule binds are summarized in Table 12.

TABLE 28: Amino acid sequences of OX40 antigens.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:54 human OX40 (Homo sapiens)	MCVGARRLGR GPCAALLLLG LGLSTVTGLH CVGDTYPSND RCCHECRPGN GMVSRCSRSQ NTVCRPCGPG FYNDVVSSKP CKPCTWCNLR SGSERKQLCT ATQDTVCRCR AGTQPLDSYK PGVDCAPCPP GHFSPGDNQA CKPWTNCTLA GKHTLQPASN SSDAICEDRD PPATQPQETQ GPPARPITVQ PTEAWPRTSQ GPSTRPVEVP GGRAVAAILG LGLVLGLLGP LAILLALYLL RRDQRLPPDA HKPPGGGSFR TPIQEEQADA HSTLAKI	60 120 180 240 277
SEQ ID NO:55 murine OX40 (Mus musculus)	MYVWVQQPTA LLLLGLTLGV TARRLNCVKH TYPSGHKCCR ECQPGHGMVS RCDHTRDTLC HPCETGFYNE AVNYDTCKQC TQCNHRSGSE LKQNCTPTQD TVCRCRPGTQ PRQDSGYKLG VDCVPCPPGH FSPGNNQACK PWTNCTLSGK QTRHPASDSL DAVCEDRSLL ATLLWETQRP TFRPTTVQST TVWPRTSELP SPPTLVTPEG PAFAVLLGLG LGLLAPLTVL LALYLLRKAW RLPNTPKPCW GNSFRTPIQE EHTDAHFTLA KI	60 120 180 240 272

[001056] In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds human or murine OX40 with a K_D of about 100 pM or lower, binds human or murine OX40 with a K_D of about 90 pM or lower, binds human or murine OX40 with a K_D of about 80 pM or lower, binds human or murine OX40 with a K_D of about 70 pM or lower, binds human or murine OX40 with a K_D of about 60 pM or lower, binds human or murine OX40 with a K_D of about 50 pM or lower, binds human or murine OX40 with a K_D of about 40 pM or lower, or binds human or murine OX40 with a K_D of about 30 pM or lower.

[001057] In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 7.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 8×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 8.5×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 9×10^5 1/M·s or faster, binds to human or murine OX40 with a k_{assoc} of about 9.5×10^5 1/M·s or faster, or binds to human or murine OX40 with a k_{assoc} of about 1×10^6 1/M·s or faster.

[001058] In some embodiments, the compositions, processes and methods described include a OX40 agonist that binds to human or murine OX40 with a k_{dissoc} of about 2×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.1×10^{-5} 1/s or slower , binds to human or murine OX40 with a k_{dissoc} of about 2.2×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.3×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.4×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.5×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.6×10^{-5} 1/s or slower or binds to human or murine OX40 with a k_{dissoc} of about 2.7×10^{-5} 1/s or slower, binds to human or murine OX40 with a k_{dissoc} of about 2.8×10^{-5} 1/s or slower, binds

to human or murine OX40 with a k_{dissoc} of about 2.9×10^{-5} 1/s or slower, or binds to human or murine OX40 with a k_{dissoc} of about 3×10^{-5} 1/s or slower.

[001059] In some embodiments, the compositions, processes and methods described include OX40 agonist that binds to human or murine OX40 with an IC_{50} of about 10 nM or lower, binds to human or murine OX40 with an IC_{50} of about 9 nM or lower, binds to human or murine OX40 with an IC_{50} of about 8 nM or lower, binds to human or murine OX40 with an IC_{50} of about 7 nM or lower, binds to human or murine OX40 with an IC_{50} of about 6 nM or lower, binds to human or murine OX40 with an IC_{50} of about 5 nM or lower, binds to human or murine OX40 with an IC_{50} of about 4 nM or lower, binds to human or murine OX40 with an IC_{50} of about 3 nM or lower, binds to human or murine OX40 with an IC_{50} of about 2 nM or lower, or binds to human or murine OX40 with an IC_{50} of about 1 nM or lower.

[001060] In some embodiments, the OX40 agonist is tavolixizumab, also known as MEDI0562 or MEDI-0562. Tavolixizumab is available from the MedImmune subsidiary of AstraZeneca, Inc. Tavolixizumab is immunoglobulin G1-kappa, anti-[*Homo sapiens* TNFRSF4 (tumor necrosis factor receptor (TNFR) superfamily member 4, OX40, CD134)], humanized and chimeric monoclonal antibody. The amino acid sequences of tavolixizumab are set forth in Table 13. Tavolixizumab comprises N-glycosylation sites at positions 301 and 301'', with fucosylated complex bi-antennary CHO-type glycans; heavy chain intrachain disulfide bridges at positions 22-95 (V_H-V_L), 148-204 (C_H1-C_L), 265-325 (C_H2) and 371-429 (C_H3) (and at positions 22''-95'', 148''-204'', 265''-325'', and 371''-429''); light chain intrachain disulfide bridges at positions 23'-88' (V_H-V_L) and 134'-194' (C_H1-C_L) (and at positions 23'''-88''' and 134'''-194'''); interchain heavy chain-heavy chain disulfide bridges at positions 230-230'' and 233-233''; and interchain heavy chain-light chain disulfide bridges at 224-214' and 224''-214'''. Current clinical trials of tavolixizumab in a variety of solid tumor indications include U.S. National Institutes of Health clinicaltrials.gov identifiers NCT02318394 and NCT02705482.

[001061] In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:56 and a light chain given by SEQ ID NO:57. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist

comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:56 and SEQ ID NO:57, respectively.

[001062] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of tavolixizumab. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:58, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:59, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively. In an embodiment, an OX40 agonist comprises an scFv antibody comprising V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59.

[001063] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:60, SEQ ID NO:61, and SEQ ID NO:62, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:63, SEQ ID NO:64, and SEQ ID NO:65, respectively, and conservative amino acid substitutions thereof.

[001064] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to tavolixizumab. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising

an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is tavolixizumab.

TABLE 29: Amino acid sequences for OX40 agonist antibodies related to tavolixizumab.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:56 heavy chain for tavolixizumab	QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVTVS SASTKGPSVF PLAPSSKSTS GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ TYICNVNHKP SNTKVDKRVE PKSCDKTHTC PPCPAPELLG GPSVFLFPPK PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP QVYTLPPSRE EMTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K	60 120 180 240 300 360 420 451
SEQ ID NO:57 light chain for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	60 120 180 214
SEQ ID NO:58 heavy chain variable region for tavolixizumab	QVQLQESGPG LVKPSQTLSL TCAVYGGSFS SGYWNWIRKH PGKGLEYIGY ISYNGITYHN PSLKSRITIN RDTSKNQYSL QLNSVTPEDT AVYYCARYKY DYDGGHAMDY WGQGTLVT	60 118
SEQ ID NO:59 light chain variable region for tavolixizumab	DIQMTQSPSS LSASVGDRVT ITCRASQDIS NYLNWYQQKP GKAPKLLIYY TSKLHSGVPS RFSGSGSGTD YTLTISSLQP EDFATYYCQQ GSALPWTFGQ GTKVEIKR	60 108

SEQ ID NO:60 heavy chain CDR1 for tavolixizumab	GSFSSGYWN	9
SEQ ID NO:61 heavy chain CDR2 for tavolixizumab	YIGYISYNGI TYH	13
SEQ ID NO:62 heavy chain CDR3 for tavolixizumab	RYKYDYDGGH AMDY	14
SEQ ID NO:63 light chain CDR1 for tavolixizumab	QDISNYLN	8
SEQ ID NO:64 light chain CDR2 for tavolixizumab	LLIYYTSKLH S	11
SEQ ID NO:65 light chain CDR3 for tavolixizumab	QQGSALPW	8

[001065] In some embodiments, the OX40 agonist is 11D4, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 11D4 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 11D4 are set forth in Table 14.

[001066] In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:66 and a light chain given by SEQ ID NO:67. In an embodiment, a OX40 agonist comprises heavy and light chains having the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:66 and SEQ ID NO:67, respectively.

[001067] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 11D4. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:68, and the OX40 agonist light

chain variable region (V_L) comprises the sequence shown in SEQ ID NO:69, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively.

[001068] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:70, SEQ ID NO:71, and SEQ ID NO:72, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:73, SEQ ID NO:74, and SEQ ID NO:75, respectively, and conservative amino acid substitutions thereof.

[001069] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 11D4. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or

more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 11D4.

TABLE 30: Amino acid sequences for OX40 agonist antibodies related to 11D4.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:66 heavy chain for 11D4	EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSSAS TKGPSVFPLA PCSRSTSEST AALGCLVKDY FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSNFGTQTYT CNVDHKPSNT KVDKTVERKC CVECPPCPAP PVAGPSVFLF PPKPKDTLMI SRTPEVTCVV VDVSHEDPEV QFNWYVDGVE VHNAKTKPRE EQFNSTFRVV SVLTVVHQDW LNGKEYKCKV SNKGLPAPIE KTISKTKGQP REPQVYTLPP SREEMTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPMLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPGK	60 120 180 240 300 360 420 444
SEQ ID NO:67 light chain for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC	60 120 180 214
SEQ ID NO:68 heavy chain variable region for 11D4	EVQLVESGGG LVQPGGSLRL SCAASGFTFS SYSMNWVRQA PGKGLEWVSY ISSSSSTIDY ADSVKGRFTI SRDNAKNSLY LQMNSLRDED TAVYYCARES GWYLFDYWGQ GTLVTVSS	60 118
SEQ ID NO:69 light chain variable region for 11D4	DIQMTQSPSS LSASVGDRVT ITCRASQGIS SWLAWYQQKP EKAPKSLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ YNSYPPTFGG GTKVEIK	60 107
SEQ ID NO:70 heavy chain CDR1 for 11D4	SYSMN	5
SEQ ID NO:71 heavy chain CDR2 for 11D4	YISSSSSTID YADSVKG	17
SEQ ID NO:72 heavy chain CDR3 for 11D4	ESGWYLFDY	9
SEQ ID NO:73 light chain CDR1 for 11D4	RASQGISSWL A	11
SEQ ID NO:74 light chain CDR2 for 11D4	AASSLQS	7
SEQ ID NO:75 light chain CDR3 for 11D4	QQYNSYPPT	9

[001070] In some embodiments, the OX40 agonist is 18D8, which is a fully human antibody available from Pfizer, Inc. The preparation and properties of 18D8 are described in U.S. Patent Nos. 7,960,515; 8,236,930; and 9,028,824, the disclosures of which are incorporated by reference herein. The amino acid sequences of 18D8 are set forth in Table 15.

[001071] In an embodiment, a OX40 agonist comprises a heavy chain given by SEQ ID NO:76 and a light chain given by SEQ ID NO:77. In an embodiment, a OX40 agonist

comprises heavy and light chains having the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively, or antigen binding fragments, Fab fragments, single-chain variable fragments (scFv), variants, or conjugates thereof. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 99% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 98% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 97% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 96% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively. In an embodiment, a OX40 agonist comprises heavy and light chains that are each at least 95% identical to the sequences shown in SEQ ID NO:76 and SEQ ID NO:77, respectively.

[001072] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of 18D8. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:78, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:79, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively.

[001073] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:80, SEQ ID NO:81, and SEQ ID NO:82, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:83, SEQ ID NO:84, and SEQ ID NO:85, respectively, and conservative amino acid substitutions thereof.

[001074] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to 18D8. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.,* 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is 18D8.

TABLE 31: Amino acid sequences for OX40 agonist antibodies related to 18D8.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:76 heavy chain for 18D8	EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV TVSSASTKGP SVFPLAPCSR STSESTAALG CLVKDYFPEP VTVSWNSGAL TSGVHTFPAV LQSSGLYSLS SVVTVPSSNF GTQTYTCNVD HKPSNTKVDK TVERKCCVEC PPCPAPPVAG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVQFNW YVDGVEVHNA KTKPREEQFN STFRVVSVLT VVHQDWLNGK EYKCKVSNKG LPAPIEKTIS KTKGQPREPQ VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPM LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPGK	60 120 180 240 300 360 420 450
SEQ ID NO:77 light chain for 18D8	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC	60 120 180 213
SEQ ID NO:78 heavy chain variable region for 18D8	EVQLVESGGG LVQPGRSLRL SCAASGFTFD DYAMHWVRQA PGKGLEWVSG ISWNSGSIGY ADSVKGRFTI SRDNAKNSLY LQMNSLRAED TALYYCAKDQ STADYYFYYG MDVWGQGTTV TVSS	60 120 124
SEQ ID NO:79 light chain	EIVVTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPTFGQG TKVEIK	60 106

variable region for 18D8		
SEQ ID NO:80 heavy chain CDR1 for 18D8	DYAMH	5
SEQ ID NO:81 heavy chain CDR2 for 18D8	GISWNSGSIG YADSVKG	17
SEQ ID NO:82 heavy chain CDR3 for 18D8	DQSTADYYFY YGMDV	15
SEQ ID NO:83 light chain CDR1 for 18D8	RASQSVSSYL A	11
SEQ ID NO:84 light chain CDR2 for 18D8	DASNRAT	7
SEQ ID NO:85 light chain CDR3 for 18D8	QQRSNWPT	8

[001075] In some embodiments, the OX40 agonist is Hu119-122, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu119-122 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu119-122 are set forth in Table 16.

[001076] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu119-122. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:86, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:87, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively.

[001077] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:88, SEQ ID NO:89, and SEQ ID NO:90, respectively, and conservative amino acid substitutions thereof, and light chain

CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:91, SEQ ID NO:92, and SEQ ID NO:93, respectively, and conservative amino acid substitutions thereof.

[001078] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu119-122. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu119-122.

TABLE 32: Amino acid sequences for OX40 agonist antibodies related to Hu119-122.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:86 heavy chain variable region for Hu119-122	EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS	60 120
SEQ ID NO:87 light chain variable region for Hu119-122	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K	60 111
SEQ ID NO:88 heavy chain CDR1 for Hu119-122	SHDMS	5

SEQ ID NO:89 heavy chain CDR2 for Hu119-122	AINSDGGSTY YPDTMER	17
SEQ ID NO:90 heavy chain CDR3 for Hu119-122	HYDDYYAWFA Y	11
SEQ ID NO:91 light chain CDR1 for Hu119-122	RASKSVSTSG YSYMH	15
SEQ ID NO:92 light chain CDR2 for Hu119-122	LASNLES	7
SEQ ID NO:93 light chain CDR3 for Hu119-122	QHSRELPLT	9

[001079] In some embodiments, the OX40 agonist is Hu106-222, which is a humanized antibody available from GlaxoSmithKline plc. The preparation and properties of Hu106-222 are described in U.S. Patent Nos. 9,006,399 and 9,163,085, and in International Patent Publication No. WO 2012/027328, the disclosures of which are incorporated by reference herein. The amino acid sequences of Hu106-222 are set forth in Table 17.

[001080] In an embodiment, the OX40 agonist comprises the heavy and light chain CDRs or variable regions (VRs) of Hu106-222. In an embodiment, the OX40 agonist heavy chain variable region (V_H) comprises the sequence shown in SEQ ID NO:94, and the OX40 agonist light chain variable region (V_L) comprises the sequence shown in SEQ ID NO:95, and conservative amino acid substitutions thereof. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 99% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 98% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 97% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 96% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively. In an embodiment, a OX40 agonist comprises V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively.

[001081] In an embodiment, a OX40 agonist comprises heavy chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:96, SEQ ID NO:97, and SEQ ID NO:98, respectively, and conservative amino acid substitutions thereof, and light chain CDR1, CDR2 and CDR3 domains having the sequences set forth in SEQ ID NO:99, SEQ ID NO:100, and SEQ ID NO:101, respectively, and conservative amino acid substitutions thereof.

[001082] In an embodiment, the OX40 agonist is a OX40 agonist biosimilar monoclonal antibody approved by drug regulatory authorities with reference to Hu106-222. In an embodiment, the biosimilar monoclonal antibody comprises an OX40 antibody comprising an amino acid sequence which has at least 97% sequence identity, *e.g.*, 97%, 98%, 99% or 100% sequence identity, to the amino acid sequence of a reference medicinal product or reference biological product and which comprises one or more post-translational modifications as compared to the reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the one or more post-translational modifications are selected from one or more of: glycosylation, oxidation, deamidation, and truncation. In some embodiments, the biosimilar is a OX40 agonist antibody authorized or submitted for authorization, wherein the OX40 agonist antibody is provided in a formulation which differs from the formulations of a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. The OX40 agonist antibody may be authorized by a drug regulatory authority such as the U.S. FDA and/or the European Union's EMA. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222. In some embodiments, the biosimilar is provided as a composition which further comprises one or more excipients, wherein the one or more excipients are the same or different to the excipients comprised in a reference medicinal product or reference biological product, wherein the reference medicinal product or reference biological product is Hu106-222.

TABLE 33: Amino acid sequences for OX40 agonist antibodies related to Hu106-222.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:94 heavy chain variable region for Hu106-222	QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV SS	60 120 122
SEQ ID NO:95 light chain variable region for Hu106-222	DIQMTQSPSS LSASVGDRVT ITCKASQDVS TAVAWYQQKP GKAPKLLIYS ASYLYTGVPS RFSGSGSGTD FTFTISSLQP EDIATYYCQQ HYSTPRTFGQ GTKLEIK	60 107
SEQ ID NO:96 heavy chain CDR1 for Hu106-222	DYSMH	5
SEQ ID NO:97 heavy chain CDR2 for Hu106-222	WINTETGEPT YADDFKG	17

SEQ ID NO:98 heavy chain CDR3 for Hu106-222	PYYDYVSYYA MDY	13
SEQ ID NO:99 light chain CDR1 for Hu106-222	KASQDVSTAV A	11
SEQ ID NO:100 light chain CDR2 for Hu106-222	SASYLYT	7
SEQ ID NO:101 light chain CDR3 for Hu106-222	QQHYSTPRT	9

[001083] In some embodiments, the OX40 agonist antibody is MEDI6469 (also referred to as 9B12). MEDI6469 is a murine monoclonal antibody. Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585. In some embodiments the OX40 agonist is an antibody produced by the 9B12 hybridoma, deposited with Biovest Inc. (Malvern, MA, USA), as described in Weinberg, *et al.*, *J. Immunother*. **2006**, *29*, 575-585, the disclosure of which is hereby incorporated by reference in its entirety. In some embodiments, the antibody comprises the CDR sequences of MEDI6469. In some embodiments, the antibody comprises a heavy chain variable region sequence and/or a light chain variable region sequence of MEDI6469.

[001084] In an embodiment, the OX40 agonist is L106 BD (Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises the CDRs of antibody L106 (BD Pharmingen Product #340420). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody L106 (BD Pharmingen Product #340420). In an embodiment, the OX40 agonist is ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises the CDRs of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In some embodiments, the OX40 agonist comprises a heavy chain variable region sequence and/or a light chain variable region sequence of antibody ACT35 (Santa Cruz Biotechnology, Catalog #20073). In an embodiment, the OX40 agonist is the murine monoclonal antibody anti-mCD134/mOX40 (clone OX86), commercially available from InVivoMAb, BioXcell Inc, West Lebanon, NH.

[001085] In an embodiment, the OX40 agonist is selected from the OX40 agonists described in International Patent Application Publication Nos. WO 95/12673, WO 95/21925, WO 2006/121810, WO 2012/027328, WO 2013/028231, WO 2013/038191, and WO 2014/148895; European Patent Application EP 0672141; U.S. Patent Application Publication Nos. US 2010/136030, US 2014/377284, US 2015/190506, and US 2015/132288 (including clones 20E5 and 12H3); and U.S. Patent Nos. 7,504,101, 7,550,140, 7,622,444, 7,696,175,

7,960,515, 7,961,515, 8,133,983, 9,006,399, and 9,163,085, the disclosure of each of which is incorporated herein by reference in its entirety.

[001086] In an embodiment, the OX40 agonist is an OX40 agonistic fusion protein as depicted in Structure I-A (C-terminal Fc-antibody fragment fusion protein) or Structure I-B (N-terminal Fc-antibody fragment fusion protein), or a fragment, derivative, conjugate, variant, or biosimilar thereof. The properties of structures I-A and I-B are described above and in U.S. Patent Nos. 9,359,420, 9,340,599, 8,921,519, and 8,450,460, the disclosures of which are incorporated by reference herein. Amino acid sequences for the polypeptide domains of structure I-A are given in Table 9. The Fc domain preferably comprises a complete constant domain (amino acids 17-230 of SEQ ID NO:31) the complete hinge domain (amino acids 1-16 of SEQ ID NO:31) or a portion of the hinge domain (*e.g.*, amino acids 4-16 of SEQ ID NO:31). Preferred linkers for connecting a C-terminal Fc-antibody may be selected from the embodiments given in SEQ ID NO:32 to SEQ ID NO:41, including linkers suitable for fusion of additional polypeptides. Likewise, amino acid sequences for the polypeptide domains of structure I-B are given in Table 10. If an Fc antibody fragment is fused to the N-terminus of an TNRFSF fusion protein as in structure I-B, the sequence of the Fc module is preferably that shown in SEQ ID NO:42, and the linker sequences are preferably selected from those embodiments set forth in SED ID NO:43 to SEQ ID NO:45.

[001087] In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains selected from the group consisting of a variable heavy chain and variable light chain of tavolixizumab, a variable heavy chain and variable light chain of 11D4, a variable heavy chain and variable light chain of 18D8, a variable heavy chain and variable light chain of Hu119-122, a variable heavy chain and variable light chain of Hu106-222, a variable heavy chain and variable light chain selected from the variable heavy chains and variable light chains described in Table 17, any combination of a variable heavy chain and variable light chain of the foregoing, and fragments, derivatives, conjugates, variants, and biosimilars thereof.

[001088] In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising an OX40L sequence. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:102. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a soluble OX40L sequence. In an

embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:103. In an embodiment, a OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains comprising a sequence according to SEQ ID NO:104.

[001089] In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:58 and SEQ ID NO:59, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:68 and SEQ ID NO:69, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:78 and SEQ ID NO:79, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:86 and SEQ ID NO:87, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the sequences shown in SEQ ID NO:94 and SEQ ID NO:95, respectively, wherein the V_H and V_L domains are connected by a linker. In an embodiment, an OX40 agonist fusion protein according to structures I-A or I-B comprises one or more OX40 binding domains that is a scFv domain comprising V_H and V_L regions that are each at least 95% identical to the V_H and V_L sequences given in Table 14, wherein the V_H and V_L domains are connected by a linker.

TABLE 34: Additional polypeptide domains useful as OX40 binding domains in fusion proteins (*e.g.*, structures I-A and I-B) or as scFv OX40 agonist antibodies.

Identifier	Sequence (One-Letter Amino Acid Symbols)	
SEQ ID NO:102 OX40L	MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL QVSHRYPRIQ SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYFS QEVNISLHYQ KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL ILIHQNPGEF CVL	60 120 180 183

SEQ ID NO:103 OX40L soluble domain	SHRYPRIQSI KVQFTEYKKE KGFILTSQKE DEIMKVQNNS VIINCDGFYL ISLKGYFSQE VNISLHYQKD EEPLFQLKKV RSVNSLMVAS LTYKDKVYLN VTTDNTSLDD FHVNGGELIL IHQNPGEFCV L	60 120 131
SEQ ID NO:104 OX40L soluble domain (alternative)	YPRIQSIKVQ FTEYKKEKGF ILTSQKEDEI MKVQNNSVII NCDGFYLISL KGYFSQEVNI SLHYQKDEEP LFQLKKVRSV NSLMVASLTY KDKVYLNVTT DNTSLDDFHV NGGELILIHQ NPGEFCVL	60 120 128
SEQ ID NO:105 variable heavy chain for 008	EVQLVESGGG LVQPGGSLRL SCAASGFTFS NYTMNWVRQA PGKGLEWVSA ISGSGGSTYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YSQVHYALDY WGQGTLVTVS	60 120
SEQ ID NO:106 variable light chain for 008	DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK	60 108
SEQ ID NO:107 variable heavy chain for 011	EVQLVESGGG VVQPGRSLRL SCAASGFTFS DYTMNWVRQA PGKGLEWVSS ISGGSTYYAD SRKGRFTISR DNSKNTLYLQ MNNLRAEDTA VYYCARDRYF RQQNAFDYWG QGTLVTVSSA	60 120
SEQ ID NO:108 variable light chain for 011	DIVMTQSPDS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKAGQSPQ LLIYLGSNRA SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYYNHP TTFGQGTK	60 108
SEQ ID NO:109 variable heavy chain for 021	EVQLVESGGG LVQPRGSLRL SCAASGFTFS SYAMNWVRQA PGKGLEWVAV ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRAED TAVYYCAKDR YITLPNALDY WGQGTLVTVS	60 120
SEQ ID NO:110 variable light chain for 021	DIQMTQSPVS LPVTPGEPAS ISCRSSQSLL HSNGYNYLDW YLQKPGQSPQ LLIYLGSNRA SGVPDRFSGS GSGTDFTLKI SRVEAEDVGV YYCQQYKSNP PTFGQGTK	60 108
SEQ ID NO:111 variable heavy chain for 023	EVQLVESGGG LVHPGGSLRL SCAGSGFTFS SYAMHWVRQA PGKGLEWVSA IGTGGGTYYA DSVMGRFTIS RDNSKNTLYL QMNSLRAEDT AVYYCARYDN VMGLYWFDYW GQGTLVTVSS	60 120
SEQ ID NO:112 variable light chain for 023	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYYCQQ RSNWPPAFGG GTKVEIKR	60 108
SEQ ID NO:113 heavy chain variable region	EVQLQQSGPE LVKPGASVKM SCKASGYTFT SYVMHWVKQK PGQGLEWIGY INPYNDGTKY NEKFKGKATL TSDKSSSTAY MELSSLTSED SAVYYCANYY GSSLSMDYWG QGTSVTVSS	60 119
SEQ ID NO:114 light chain variable region	DIQMTQTTSS LSASLGDRVT ISCRASQDIS NYLNWYQQKP DGTVKLLIYY TSRLHSGVPS RFSGSGSGTD YSLTISNLEQ EDIATYFCQQ GNTLPWTFGG GTKLEIKR	60 108
SEQ ID NO:115 heavy chain variable region	EVQLQQSGPE LVKPGASVKI SCKTSGYTFK DYTMHWVKQS HGKSLEWIGG IYPNNGGSTY NQNFKDKATL TVDKSSSTAY MEFRSLTSED SAVYYCARMG YHGPHLDFDV WGAGTTVTVS P	60 120 121
SEQ ID NO:116 light chain variable region	DIVMTQSHKF MSTSLGDRVS ITCKASQDVG AAVAWYQQKP GQSPKLLIYW ASTRHTGVPD RFTGGGSGTD FTLTISNVQS EDLTDYFCQQ YINYPLTFGG GTKLEIKR	60 108
SEQ ID NO:117 heavy chain variable region of humanized antibody	QIQLVQSGPE LKKPGETVKI SCKASGYTFT DYSMHWVKQA PGKGLKWMGW INTETGEPTY ADDFKGRFAF SLETSASTAY LQINNLKNED TATYFCANPY YDYVSYYAMD YWGHGTSVTV SS	60 120 122
SEQ ID NO:118 heavy chain variable region of humanized antibody	QVQLVQSGSE LKKPGASVKV SCKASGYTFT DYSMHWVRQA PGQGLKWMGW INTETGEPTY ADDFKGRFVF SLDTSVSTAY LQISSLKAED TAVYYCANPY YDYVSYYAMD YWGQGTTVTV SS	60 120 122
SEQ ID NO:119 light chain variable region of humanized antibody	DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK	60 107
SEQ ID NO:120 light chain variable region of humanized antibody	DIVMTQSHKF MSTSVRDRVS ITCKASQDVS TAVAWYQQKP GQSPKLLIYS ASYLYTGVPD RFTGSGSGTD FTFTISSVQA EDLAVYYCQQ HYSTPRTFGG GTKLEIK	60 107
SEQ ID NO:121 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGESLKL SCESNEYEFP SHDMSWVRKT PEKRLELVAA INSDGGSTYY PDTMERRFII SRDNTKKTLY LQMSSLRSED TALYYCARHY DDYYAWFAYW GQGTLVTVSA	60 120
SEQ ID NO:122 heavy chain variable region of humanized antibody	EVQLVESGGG LVQPGGSLRL SCAASEYEFP SHDMSWVRQA PGKGLELVAA INSDGGSTYY PDTMERRFTI SRDNAKNSLY LQMNSLRAED TAVYYCARHY DDYYAWFAYW GQGTMVTVSS	60 120
SEQ ID NO:123 light chain	DIVLTQSPAS LAVSLGQRAT ISCRASKSVS TSGYSYMHWY QQKPGQPPKL LIYLASNLES GVPARFSGSG SGTDFTLNIH PVEEEDAATY YCQHSRELPL TFGAGTKLEL K	60 111

variable region of humanized antibody		
SEQ ID NO:124 light chain variable region of humanized antibody	EIVLTQSPAT LSLSPGERAT LSCRASKSVS TSGYSYMHWY QQKPGQAPRL LIYLASNLES GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRELPL TFGGGTKVEI K	60 111
SEQ ID NO:125 heavy chain variable region	MYLGLNYVFI VFLLNGVQSE VKLEESGGGL VQPGGSMKLS CAASGFTFSD AWMDWVRQSP EKGLEWVAEI RSKANNHATY YAESVNGRFT ISRDDSKSSV YLQMNSLRAE DTGIYYCTWG EVFYFDYWGQ GTTLTVSS	60 120 138
SEQ ID NO:126 light chain variable region	MRPSIQFLGL LLFWLHGAQC DIQMTQSPSS LSASLGGKVT ITCKSSQDIN KYIAWYQHKP GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLLTFGAG TKLELK	60 120 126

[001090] In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, and wherein the additional domain is a Fab or Fc fragment domain. In an embodiment, the OX40 agonist is a OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble OX40 binding domain, (ii) a first peptide linker, (iii) a second soluble OX40 binding domain, (iv) a second peptide linker, and (v) a third soluble OX40 binding domain, further comprising an additional domain at the N-terminal and/or C-terminal end, wherein the additional domain is a Fab or Fc fragment domain wherein each of the soluble OX40 binding domains lacks a stalk region (which contributes to trimerisation and provides a certain distance to the cell membrane, but is not part of the OX40 binding domain) and the first and the second peptide linkers independently have a length of 3-8 amino acids.

[001091] In an embodiment, the OX40 agonist is an OX40 agonistic single-chain fusion polypeptide comprising (i) a first soluble tumor necrosis factor (TNF) superfamily cytokine domain, (ii) a first peptide linker, (iii) a second soluble TNF superfamily cytokine domain, (iv) a second peptide linker, and (v) a third soluble TNF superfamily cytokine domain, wherein each of the soluble TNF superfamily cytokine domains lacks a stalk region and the first and the second peptide linkers independently have a length of 3-8 amino acids, and wherein the TNF superfamily cytokine domain is an OX40 binding domain.

[001092] In some embodiments, the OX40 agonist is MEDI6383. MEDI6383 is an OX40 agonistic fusion protein and can be prepared as described in U.S. Patent No. 6,312,700, the disclosure of which is incorporated by reference herein.

[001093] In an embodiment, the OX40 agonist is an OX40 agonistic scFv antibody comprising any of the foregoing V_H domains linked to any of the foregoing V_L domains.

[001094] In an embodiment, the OX40 agonist is Creative Biolabs OX40 agonist monoclonal antibody MOM-18455, commercially available from Creative Biolabs, Inc., Shirley, NY, USA.

[001095] In an embodiment, the OX40 agonist is OX40 agonistic antibody clone Ber-ACT35 commercially available from BioLegend, Inc., San Diego, CA, USA.

I. Optional Cell Viability Analyses

[001096] Optionally, a cell viability assay can be performed after the priming first expansion (sometimes referred to as the initial bulk expansion), using standard assays known in the art. Thus, in certain embodiments, the method comprises performing a cell viability assay subsequent to the priming first expansion. For example, a trypan blue exclusion assay can be done on a sample of the bulk TILs, which selectively labels dead cells and allows a viability assessment. Other assays for use in testing viability can include but are not limited to the Alamar blue assay; and the MTT assay.

1. Cell Counts, Viability, Flow Cytometry

[001097] In some embodiments, cell counts and/or viability are measured. The expression of markers such as but not limited CD3, CD4, CD8, and CD56, as well as any other disclosed or described herein, can be measured by flow cytometry with antibodies, for example but not limited to those commercially available from BD Bio-sciences (BD Biosciences, San Jose, CA) using a FACSCanto™ flow cytometer (BD Biosciences). The cells can be counted manually using a disposable c-chip hemocytometer (VWR, Batavia, IL) and viability can be assessed using any method known in the art, including but not limited to trypan blue staining. The cell viability can also be assayed based on USSN 15/863,634, incorporated by reference herein in its entirety. Cell viability can also be assayed based on U.S. Patent Publication No. 2018/0280436 or International Patent Publication No. WO/2018/081473, both of which are incorporate herein in their entireties for all purposes.

[001098] In some cases, the bulk TIL population can be cryopreserved immediately, using the protocols discussed below. Alternatively, the bulk TIL population can be subjected to REP and then cryopreserved as discussed below. Similarly, in the case where genetically modified TILs will be used in therapy, the bulk or REP TIL populations can be subjected to genetic modifications for suitable treatments.

2. Cell Cultures

[001099] In an embodiment, a method for expanding TILs, including those discussed above as well as exemplified in Figure 8, in particular, *e.g.*, Figure 8B and/or Figure 8C, may include using about 5,000 mL to about 25,000 mL of cell medium, about 5,000 mL to about 10,000 mL of cell medium, or about 5,800 mL to about 8,700 mL of cell medium. In some embodiments, the media is a serum free medium. In some embodiments, the media in the priming first expansion is serum free. In some embodiments, the media in the second expansion is serum free. In some embodiments, the media in the priming first expansion and the second expansion (also referred to as rapid second expansion) are both serum free. In an embodiment, expanding the number of TILs uses no more than one type of cell culture medium. Any suitable cell culture medium may be used, *e.g.*, AIM-V cell medium (L-glutamine, 50 μM streptomycin sulfate, and 10 μM gentamicin sulfate) cell culture medium (Invitrogen, Carlsbad CA). In this regard, the inventive methods advantageously reduce the amount of medium and the number of types of medium required to expand the number of TIL. In an embodiment, expanding the number of TIL may comprise feeding the cells no more frequently than every third or fourth day. Expanding the number of cells in a gas permeable container simplifies the procedures necessary to expand the number of cells by reducing the feeding frequency necessary to expand the cells.

[001100] In an embodiment, the cell culture medium in the first and/or second gas permeable container is unfiltered. The use of unfiltered cell medium may simplify the procedures necessary to expand the number of cells. In an embodiment, the cell medium in the first and/or second gas permeable container lacks beta-mercaptoethanol (BME).

[001101] In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable container containing cell medium including IL-2, 1X antigen-presenting feeder cells, and OKT-3 for a duration of about 1 to 8 days, *e.g.*, about 7 days as a priming first expansion, or about 8 days as a priming first expansion; transferring the TILs to a second gas permeable container and expanding the number of TILs in the second gas permeable container containing cell medium including IL-2, 2X antigen-presenting feeder cells, and OKT-3 for a duration of about 7 to 9 days, *e.g.*, about 7 days, about 8 days, or about 9 days.

[001102] In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable

container containing cell medium including IL-2, 1X antigen-presenting feeder cells, and OKT-3 for a duration of about 1 to 7 days, *e.g.*, about 7 days as a priming first expansion; transferring the TILs to a second gas permeable container and expanding the number of TILs in the second gas permeable container containing cell medium including IL-2, 2X antigen-presenting feeder cells, and OKT-3 for a duration of about 7 to 9 days, *e.g.*, about 7 days, about 8 days, or about 9 days.

[001103] In an embodiment, the duration of the method comprising obtaining a tumor tissue sample from the mammal; culturing the tumor tissue sample in a first gas permeable container containing cell medium including IL-2, 1X antigen-presenting feeder cells, and OKT-3 for a duration of about 1 to 7 days, *e.g.*, about 7 days as a priming first expansion; transferring the TILs to a second gas permeable container and expanding the number of TILs in the second gas permeable container containing cell medium including IL-2, 2X antigen-presenting feeder cells, and OKT-3 for a duration of about 7 to 10 days, *e.g.*, about 7 days, about 8 days, about 9 days or about 10 days.

[001104] In an embodiment, TILs are expanded in gas-permeable containers. Gas-permeable containers have been used to expand TILs using PBMCs using methods, compositions, and devices known in the art, including those described in U.S. Patent Application Publication No. 2005/0106717 A1, the disclosures of which are incorporated herein by reference. In an embodiment, TILs are expanded in gas-permeable bags. In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the Xuri Cell Expansion System W25 (GE Healthcare). In an embodiment, TILs are expanded using a cell expansion system that expands TILs in gas permeable bags, such as the WAVE Bioreactor System, also known as the Xuri Cell Expansion System W5 (GE Healthcare). In an embodiment, the cell expansion system includes a gas permeable cell bag with a volume selected from the group consisting of about 100 mL, about 200 mL, about 300 mL, about 400 mL, about 500 mL, about 600 mL, about 700 mL, about 800 mL, about 900 mL, about 1 L, about 2 L, about 3 L, about 4 L, about 5 L, about 6 L, about 7 L, about 8 L, about 9 L, and about 10 L.

[001105] In an embodiment, TILs can be expanded in G-Rex flasks (commercially available from Wilson Wolf Manufacturing). Such embodiments allow for cell populations to expand from about 5×10^5 cells/cm^2 to between 10×10^6 and 30×10^6 cells/cm^2. In an embodiment this is without feeding. In an embodiment, this is without feeding so long as medium resides at a height of about 10 cm in the G-Rex flask. In an embodiment this is without feeding but

with the addition of one or more cytokines. In an embodiment, the cytokine can be added as a bolus without any need to mix the cytokine with the medium. Such containers, devices, and methods are known in the art and have been used to expand TILs, and include those described in U.S. Patent Application Publication No. US 2014/0377739A1, International Publication No. WO 2014/210036 A1, U.S. Patent Application Publication No. us 2013/0115617 A1, International Publication No. WO 2013/188427 A1, U.S. Patent Application Publication No. US 2011/0136228 A1, U.S. Patent No. US 8,809,050 B2, International publication No. WO 2011/072088 A2, U.S. Patent Application Publication No. US 2016/0208216 A1, U.S. Patent Application Publication No. US 2012/0244133 A1, International Publication No. WO 2012/129201 A1, U.S. Patent Application Publication No. US 2013/0102075 A1, U.S. Patent No. US 8,956,860 B2, International Publication No. WO 2013/173835 A1, U.S. Patent Application Publication No. US 2015/0175966 A1, the disclosures of which are incorporated herein by reference. Such processes are also described in Jin *et al.*, *J. Immunotherapy,* **2012**, 35:283-292.

J. Optional Genetic Engineering of TILs

[001106] In some embodiments, the expanded TILs of the present invention are further manipulated before, during, or after an expansion step, including during closed, sterile manufacturing processes, each as provided herein, in order to alter protein expression in a transient manner. In some embodiments, the transiently altered protein expression is due to transient gene editing. In some embodiments, the expanded TILs of the present invention are treated with transcription factors (TFs) and/or other molecules capable of transiently altering protein expression in the TILs. In some embodiments, the TFs and/or other molecules that are capable of transiently altering protein expression provide for altered expression of tumor antigens and/or an alteration in the number of tumor antigen-specific T cells in a population of TILs.

[001107] In certain embodiments, the method comprises genetically editing a population of TILs. In certain embodiments, the method comprises genetically editing the first population of TILs, the second population of TILs and/or the third population of TILs.

[001108] In some embodiments, the present invention includes genetic editing through nucleotide insertion, such as through ribonucleic acid (RNA) insertion, including insertion of messenger RNA (mRNA) or small (or short) interfering RNA (siRNA), into a population of TILs for promotion of the expression of one or more proteins or inhibition of the expression

of one or more proteins, as well as simultaneous combinations of both promotion of one set of proteins with inhibition of another set of proteins.

[001109] In some embodiments, the expanded TILs of the present invention undergo transient alteration of protein expression. In some embodiments, the transient alteration of protein expression occurs in the bulk TIL population prior to first expansion, including, for example in the TIL population obtained from for example, Step A as indicated in Figure 8 (particularly Figure 8B and/or Figure 8C). In some embodiments, the transient alteration of protein expression occurs during the first expansion, including, for example in the TIL population expanded in for example, Step B as indicated in Figure 8 (for example Figure 8B and/or Figure 8C). In some embodiments, the transient alteration of protein expression occurs after the first expansion, including, for example in the TIL population in transition between the first and second expansion (e.g. the second population of TILs as described herein), the TIL population obtained from for example, Step B and included in Step C as indicated in Figure 8. In some embodiments, the transient alteration of protein expression occurs in the bulk TIL population prior to second expansion, including, for example in the TIL population obtained from for example, Step C and prior to its expansion in Step D as indicated in Figure 8. In some embodiments, the transient alteration of protein expression occurs during the second expansion, including, for example in the TIL population expanded in for example, Step D as indicated in Figure 8 (e.g. the third population of TILs). In some embodiments, the transient alteration of protein expression occurs after the second expansion, including, for example in the TIL population obtained from the expansion in for example, Step D as indicated in Figure 8.

[001110] In an embodiment, a method of transiently altering protein expression in 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. In an embodiment, a method of transiently altering protein expression in 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 transiently altering protein expression in 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 transiently altering protein expression in 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.

[001111] In some embodiments, transient alteration of protein expression results in an increase in Stem Memory T cells (TSCMs). TSCMs are early progenitors of antigen-experienced central memory T cells. TSCMs generally display the long-term survival, self-renewal, and multipotency abilities that define stem cells, and are generally desirable for the generation of effective TIL products. TSCM have shown enhanced anti-tumor activity compared with other T cell subsets in mouse models of adoptive cell transfer (Gattinoni *et al.* Nat Med 2009, 2011; Gattinoni, Nature Rev. Cancer, 2012; Cieri *et al.* Blood 2013). In some embodiments, transient alteration of protein expression results in a TIL population with a composition comprising a high proportion of TSCM. In some embodiments, transient alteration of protein expression results in an at least 5%, at least 10%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% increase in TSCM percentage. In some embodiments, transient alteration of protein expression results in an at least a 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, or 10-fold increase in TSCMs in the TIL population. In some embodiments, transient alteration of protein expression results in a TIL population with at least at least 5%, at least 10%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% TSCMs. In some embodiments, transient alteration of protein expression results in a therapeutic TIL population with at least at least 5%, at least 10%, at least 10%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least

45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% TSCMs.

[001112] In some embodiments, transient alteration of protein expression results in rejuvenation of antigen-experienced T-cells. In some embodiments, rejuvenation includes, for example, increased proliferation, increased T-cell activation, and/or increased antigen recognition.

[001113] In some embodiments, transient alteration of protein expression alters the expression in a large fraction of the T-cells in order to preserve the tumor-derived TCR repertoire. In some embodiments, transient alteration of protein expression does not alter the tumor-derived TCR repertoire. In some embodiments, transient alteration of protein expression maintains the tumor-derived TCR repertoire.

[001114] In some embodiments, transient alteration of protein results in altered expression of a particular gene. In some embodiments, the transient alteration of protein expression targets a gene including but not limited to PD-1 (also referred to as PDCD1 or CC279), TGFBR2, CCR4/5, CBLB (CBL-B), CISH, CCRs (chimeric co-stimulatory receptors), IL-2, IL-12, IL-15, IL-21, NOTCH 1/2 ICD, TIM3, LAG3, TIGIT, TGFβ, CCR2, CCR4, CCR5, CXCR1, CXCR2, CSCR3, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL1/CXCL8, CCL22, CCL17, CXCL1/CXCL8, VHL, CD44, PIK3CD, SOCS1, and/or cAMP protein kinase A (PKA). In some embodiments, the transient alteration of protein expression targets a gene selected from the group consisting of PD-1, TGFBR2, CCR4/5, CBLB (CBL-B), CISH, CCRs (chimeric co-stimulatory receptors), IL-2, IL-12, IL-15, IL-21, NOTCH 1/2 ICD, TIM3, LAG3, TIGIT, TGFβ, CCR2, CCR4, CCR5, CXCR1, CXCR2, CSCR3, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL1/CXCL8, CCL22, CCL17, CXCL1/CXCL8, VHL, CD44, PIK3CD, SOCS1, and/or cAMP protein kinase A (PKA). In some embodiments, the transient alteration of protein expression targets PD-1. In some embodiments, the transient alteration of protein expression targets TGFBR2. In some embodiments, the transient alteration of protein expression targets CCR4/5. In some embodiments, the transient alteration of protein expression targets CBLB. In some embodiments, the transient alteration of protein expression targets CISH. In some embodiments, the transient alteration of protein expression targets CCRs (chimeric co-stimulatory receptors). In some embodiments, the transient alteration of protein expression targets IL-2. In some embodiments, the transient alteration of protein expression targets IL-12. In some embodiments, the transient alteration of protein expression targets IL-15. In

some embodiments, the transient alteration of protein expression targets IL-21. In some embodiments, the transient alteration of protein expression targets NOTCH 1/2 ICD. In some embodiments, the transient alteration of protein expression targets TIM3. In some embodiments, the transient alteration of protein expression targets LAG3. In some embodiments, the transient alteration of protein expression targets TIGIT. In some embodiments, the transient alteration of protein expression targets TGFβ. In some embodiments, the transient alteration of protein expression targets CCR1. In some embodiments, the transient alteration of protein expression targets CCR2. In some embodiments, the transient alteration of protein expression targets CCR4. In some embodiments, the transient alteration of protein expression targets CCR5. In some embodiments, the transient alteration of protein expression targets CXCR1. In some embodiments, the transient alteration of protein expression targets CXCR2. In some embodiments, the transient alteration of protein expression targets CSCR3. In some embodiments, the transient alteration of protein expression targets CCL2 (MCP-1). In some embodiments, the transient alteration of protein expression targets CCL3 (MIP-1α). In some embodiments, the transient alteration of protein expression targets CCL4 (MIP1-β). In some embodiments, the transient alteration of protein expression targets CCL5 (RANTES). In some embodiments, the transient alteration of protein expression targets CXCL1. In some embodiments, the transient alteration of protein expression targets CXCL8. In some embodiments, the transient alteration of protein expression targets CCL22. In some embodiments, the transient alteration of protein expression targets CCL17. In some embodiments, the transient alteration of protein expression targets VHL. In some embodiments, the transient alteration of protein expression targets CD44. In some embodiments, the transient alteration of protein expression targets PIK3CD. In some embodiments, the transient alteration of protein expression targets SOCS1. In some embodiments, the transient alteration of protein expression targets cAMP protein kinase A (PKA).

[001115] In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of a chemokine receptor. In some embodiments, the chemokine receptor that is overexpressed by transient protein expression includes a receptor with a ligand that includes but is not limited to CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP1-β), CCL5 (RANTES), CXCL1, CXCL8, CCL22, and/or CCL17.

[001116] In some embodiments, the transient alteration of protein expression results in a decrease and/or reduced expression of PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, TGFβR2, and/or TGFβ (including resulting in, for example, TGFβ pathway blockade). In some embodiments, the transient alteration of protein expression results in a decrease and/or reduced expression of CBLB (CBL-B). In some embodiments, the transient alteration of protein expression results in a decrease and/or reduced expression of CISH.

[001117] In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of chemokine receptors in order to, for example, improve TIL trafficking or movement to the tumor site. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of a CCR (chimeric co-stimulatory receptor). In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of a chemokine receptor selected from the group consisting of CCR1, CCR2, CCR4, CCR5, CXCR1, CXCR2, and/or CSCR3.

[001118] In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of an interleukin. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of an interleukin selected from the group consisting of IL-2, IL-12, IL-15, and/or IL-21.

[001119] In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of NOTCH 1/2 ICD. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of VHL. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of CD44. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of PIK3CD. In some embodiments, the transient alteration of protein expression results in increased and/or overexpression of SOCS1,

[001120] In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of cAMP protein kinase A (PKA).

[001121] In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of a molecule selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of two molecules selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF

(BR3), and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and one molecule selected from the group consisting of LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1, LAG-3, CISH, CBLB, TIM3, and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and one of LAG3, CISH, CBLB, TIM3, and combinations thereof. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and LAG3. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and CISH. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of PD-1 and CBLB. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of LAG3 and CISH. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of LAG3 and CBLB. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of CISH and CBLB. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and PD-1. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and LAG3. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and CISH. In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of TIM3 and CBLB.

[001122] In some embodiments, an adhesion molecule selected from the group consisting of CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, and combinations thereof, is inserted by a gammaretroviral or lentiviral method into the first population of TILs, second population of TILs, or harvested population of TILs (*e.g.*, the expression of the adhesion molecule is increased).

[001123] In some embodiments, the transient alteration of protein expression results in decreased and/or reduced expression of a molecule selected from the group consisting of PD-1, LAG3, TIM3, CTLA-4, TIGIT, CISH, TGFβR2, PKA, CBLB, BAFF (BR3), and combinations thereof, and increased and/or enhanced expression of CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, and combinations thereof. In some embodiments, the transient

alteration of protein expression results in decreased and/or reduced expression of a molecule selected from the group consisting of PD-1, LAG3, TIM3, CISH, CBLB, and combinations thereof, and increased and/or enhanced expression of CCR2, CCR4, CCR5, CXCR2, CXCR3, CX3CR1, and combinations thereof.

[001124] In some embodiments, there is a reduction in expression of about 5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 85%, about 90%, or about 95%. In some embodiments, there is a reduction in expression of at least about 80%. In some embodiments, there is a reduction in expression of at least about 85%, In some embodiments, there is a reduction in expression of at least about 90%. In some embodiments, there is a reduction in expression of at least about 95%. In some embodiments, there is a reduction in expression of at least about 99%.

[001125] In some embodiments, there is an increase in expression of about 5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 75%, about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 80%, about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 85%, about 90%, or about 95%. In some embodiments, there is an increase in expression of at least about 80%. In some embodiments, there is an increase in expression of at least about 85%, In some embodiments, there is an increase in expression of at least about 90%. In some embodiments, there is an increase in expression of at least about 95%. In some embodiments, there is an increase in expression of at least about 99%.

[001126] In some embodiments, transient alteration of protein expression is induced by treatment of the TILs with transcription factors (TFs) and/or other molecules capable of transiently altering protein expression in the TILs. In some embodiments, the SQZ vector-free microfluidic platform is employed for intracellular delivery of the transcription factors (TFs) and/or other molecules capable of transiently altering protein expression. Such methods demonstrating the ability to deliver proteins, including transcription factors, to a variety of primary human cells, including T cells (Sharei *et al.* PNAS 2013, as well as Sharei *et al.* PLOS ONE 2015 and Greisbeck *et al.* J. Immunology vol. 195, 2015) have been described; see, for example, International Patent Publications WO 2013/059343A1, WO 2017/008063A1, and WO 2017/123663A1, all of which are incorporated by reference herein in their entireties. Such methods as described in International Patent Publications WO 2013/059343A1, WO 2017/008063A1, and WO 2017/123663A1 can be employed with the present invention in order to expose a population of TILs to transcription factors (TFs) and/or other molecules capable of inducing transient protein expression, wherein said TFs and/or other molecules capable of inducing transient protein expression provide for increased expression of tumor antigens and/or an increase in the number of tumor antigen-specific T cells in the population of TILs, thus resulting in reprogramming of the TIL population and an increase in therapeutic efficacy of the reprogrammed TIL population as compared to a non-reprogrammed TIL population. In some embodiments, the reprogramming results in an increased subpopulation of effector T cells and/or central memory T cells relative to the starting or prior population (*i.e.*, prior to reprogramming) population of TILs, as described herein.

[001127] In some embodiments, the transcription factor (TF) includes but is not limited to TCF-1, NOTCH 1/2 ICD, and/or MYB. In some embodiments, the transcription factor (TF) is TCF-1. In some embodiments, the transcription factor (TF) is NOTCH 1/2 ICD. In some embodiments, the transcription factor (TF) is MYB. In some embodiments, the transcription factor (TF) is administered with induced pluripotent stem cell culture (iPSC), such as the commercially available KNOCKOUT Serum Replacement (Gibco/ThermoFisher), to induce additional TIL reprogramming. In some embodiments, the transcription factor (TF) is administered with an iPSC cocktail to induce additional TIL reprogramming. In some embodiments, the transcription factor (TF) is administered without an iPSC cocktail. In some embodiments, reprogramming results in an increase in the percentage of TSCMs. In some embodiments, reprogramming results in an increase in the percentage of TSCMs by about

5%, about 10%, about 10%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95% TSCMs.

[001128] In some embodiments, a method of transient altering protein expression, as described above, may be combined with 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 certain embodiments, the method comprises a step of genetically modifying a population of TILs. In certain embodiments, the method comprises genetically modifying the first population of TILs, the second population of TILs and/or the third population of TILs. 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.

[001129] In some embodiments, transient alteration of protein expression is a reduction in expression induced by self-delivering RNA interference (sdRNA), which is a chemically-synthesized asymmetric siRNA duplex with a high percentage of 2'-OH substitutions

(typically fluorine or -OCH_3) which comprises a 20-nucleotide antisense (guide) strand and a 13 to 15 base sense (passenger) strand conjugated to cholesterol at its 3' end using a tetraethylenglycol (TEG) linker. In some embodiments, the method comprises transient alteration of protein expression in a population of TILs, comprising the use of self-delivering RNA interference (sdRNA), which is a chemically-synthesized asymmetric siRNA duplex with a high percentage of 2'-OH substitutions (typically fluorine or -OCH_3) which comprises a 20-nucleotide antisense (guide) strand and a 13 to 15 base sense (passenger) strand conjugated to cholesterol at its 3' end using a tetraethylenglycol (TEG) linker. Methods of using sdRNA have been described in Khvorova and Watts, *Nat. Biotechnol.* **2017,** *35,* 238–248; Byrne, *et al.*, *J. Ocul. Pharmacol. Ther.* **2013,** *29,* 855-864; and Ligtenberg, *et al.*, *Mol. Therapy,* **2018,** in press, the disclosures of which are incorporated by reference herein. In an embodiment, delivery of sdRNA to a TIL population is accomplished without use of electroporation, SQZ, or other methods, instead using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of 1 μM/10,000 TILs in medium. In certain embodiments, the method comprises delivery sdRNA to a TILs population comprising exposing the TILs population to sdRNA at a concentration of 1 μM/10,000 TILs in medium for a period of between 1 to 3 days. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of 10 μM/10,000 TILs in medium. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of 50 μM/10,000 TILs in medium. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of between 0.1 μM/10,000 TILs and 50 μM/10,000 TILs in medium. In an embodiment, delivery of sdRNA to a TIL population is accomplished using a 1 to 3 day period in which a TIL population is exposed to sdRNA at a concentration of between 0.1 μM/10,000 TILs and 50 μM/10,000 TILs in medium, wherein the exposure to sdRNA is performed two, three, four, or five times by addition of fresh sdRNA to the media. Other suitable processes are described, for example, in U.S. Patent Application Publication No. US 2011/0039914 A1, US 2013/0131141 A1, and US 2013/0131142 A1, and U.S. Patent No. 9,080,171, the disclosures of which are incorporated by reference herein.

[001130] In some embodiments, sdRNA is inserted into a population of TILs during manufacturing. In some embodiments, the sdRNA encodes RNA that interferes with

DEMANDE OU BREVET VOLUMINEUX

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

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JUMBO APPLICATIONS/PATENTS

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WHAT IS CLAIMED IS:

1. A method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor infiltrating lymphocytes (TILs) comprising the steps of:

   (a) obtaining and/or receiving a first population of TILs from surgical resection, needle biopsy, core biopsy, small biopsy, or other means for obtaining a sample that contains a mixture of tumor and TIL cells from a NSCLC tumor in a patient;

   (c) contacting the tumor fragments with a first cell culture medium;

   (d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

   (e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

   (f) harvesting the third population of TILs; and

   (g) administering a therapeutically effective portion of the third population of TILs to a patient with the NSCLC;

   wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

2. The method of Claim 1, wherein the obtaing the first population of TILs comprises a multilesional sampling method.

3. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 and/or anti-PD-L2 antibody.

4. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

5. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent.

6. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

7. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

8. The method of Claims 5 to 7, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

9. The method of Claim 1, wherein the refractory NSCLC has low expression of PD-L1.

10. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

11. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

12. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

13. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

14. The method of Claim 1, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

15. The method of Claim 1, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

16. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

17. The method of Claim 1, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

18. The method of Claims 14 to 17, wherein bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane

or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

19. The method of Claim 1, wherein the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

20. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

21. The method of Claim 1, wherein the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

22. The method of Claim 1, wherein the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

23. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

24. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

25. The method of Claim 1, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

26. The method of Claim 1, wherein the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

27. The method of Claim 1, wherein the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

28. The method of Claim 1, wherein the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

29. The method of any one of Claims 1 to 28, wherein the initial expansion is performed over a period of 21 days or less.

30. The method of any one of Claims 1 to 29, wherein the initial expansion is performed over

a period of 14 days or less.

31. The method of any one of Claims 1 to 30, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

32. The method of any one of Claims 1 to 31, wherein 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 in the second cell culture medium.

33. The method of any one of Claims 1 to 32, wherein the initial expansion is performed using a gas permeable container.

34. The method of any one of Claims 1 to 33, wherein the rapid expansion is performed using a gas permeable container.

35. The method of any one of Claims 1 to 34, wherein 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.

36. The method of any one of Claims 1 to 35, wherein the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

37. The method of any one of Claims 1 to 36, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

38. The method of Claim 37, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m^2/day for two days followed by administration of fludarabine at a dose of 25 mg/m^2/day for five days.

39. The method of any one of Claims 1 to 38, further comprising the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

40. The method of Claim 39, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

41. A method of treating non-small cell lung carcinoma (NSCLC) with a population of tumor

infiltrating lymphocytes (TILs) comprising the steps of:

(a) resecting a tumor from a patient, the tumor comprising a first population of TILs;

(b) fragmenting the tumor into tumor fragments;

(c) contacting the tumor fragments with a first cell culture medium;

(d) performing an initial expansion of the first population of TILs in the first cell culture medium to obtain a second population of TILs, wherein the second population of TILs is at least 5-fold greater in number than the first population of TILs, wherein the first cell culture medium comprises IL-2;

(e) performing a rapid expansion of the second population of TILs in a second cell culture medium to obtain a third population of TILs, wherein the third population of TILs is at least 50-fold greater in number than the second population of TILs after 7 days from the start of the rapid expansion; wherein the second cell culture medium comprises IL-2, OKT-3 (anti-CD3 antibody), and optionally irradiated allogeneic peripheral blood mononuclear cells (PBMCs); and wherein the rapid expansion is performed over a period of 14 days or less;

(f) harvesting the third population of TILs; and

(g) administering a therapeutically effective portion of the third population of TILs to a patient with the NSCLC;

wherein the NSCLC is refractory to treatment with an anti-PD-1 antibody.

42. The method of Claim 41, wherein the tumor is resected from one or more tumor cites.

43. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 and/or anti-PD-L2 antibody.

44. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

45. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent.

46. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

47. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

48. The method of Claims 45 to 47, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

49. The method of Claim 41, wherein the refractory NSCLC has low expression of PD-L1.

50. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

51. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

52. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

53. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1.

54. The method of Claim 41, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

55. The method of Claim 41, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

56. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

57. The method of Claim 41, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

58. The method of Claims 54 to 57, wherein said bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

59. The method of Claim 41, wherein the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

60. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

61. The method of Claim 41, wherein the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

62. The method of Claim 41, wherein the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

63. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

64. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

65. The method of Claim 41, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

66. The method of Claim 41, wherein the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

67. The method of Claim 41, wherein the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

68. The method of Claim 41, wherein the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

69. The method of any one of Claims 41 to 68, wherein the initial expansion is performed over a period of 21 days or less.

70. The method of any one of Claims 41 to 69, wherein the initial expansion is performed over a period of 14 days or less.

71. The method of any one of Claims 41 to 70, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

72. The method of any one of Claims 41 to 71, wherein 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 in the second cell culture medium.

73. The method of any one of Claims 41 to 72, wherein the initial expansion is performed using a gas permeable container.

74. The method of any one of Claims 41 to 73, wherein the rapid expansion is performed using a gas permeable container.

75. The method of any one of Claims 41 to 74, wherein 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.

76. The method of any one of Claims 41 to 75, wherein the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

77. The method of any one of Claims 41 to 76, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

78. The method of Claim 77, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m^2/day for two days followed by administration of fludarabine at a dose of 25 mg/m^2/day for five days.

79. The method of any one of Claims 41 to 78, further comprising the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

80. The method of Claim 79, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof, administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

81. A method for treating a subject with non-small cell lung carcinoma (NSCLC), wherein the cancer is refractory to treatment with an anti-PD-1 antibody, the method comprising administering expanded tumor infiltrating lymphocytes (TILs) comprising:

    (a) obtaining and/or receiving a first population of TILs from a tumor resected from a subject by processing a tumor sample obtained from the subject 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 to produce a second population of TILs, wherein the first expansion is performed in a closed container providing a first gas-permeable surface area, wherein the first expansion is performed for about 3-14 days to obtain the second population of TILs, wherein the second population of TILs is at least 50-fold greater in number than the first population of TILs, and wherein the transition from step (b) to step (c) occurs without opening the system;

(d) performing a second expansion by supplementing the cell culture medium of the second population of TILs with additional IL-2, OKT-3, and antigen presenting cells (APCs), to produce a third population of TILs, wherein the second expansion is performed for about 7-14 days to obtain the third population of TILs, wherein the third population of TILs is a therapeutic population of TILs which comprises an increased subpopulation of effector T cells and/or central memory T cells relative to the second population of TILs, wherein the second expansion is performed in a closed container providing a second gas-permeable surface area, and wherein the transition from step (c) to step (d) occurs without opening the system;

(e) harvesting therapeutic population of TILs obtained from step (d), wherein the transition from step (d) to step (e) occurs without opening the system; and

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

(g) cryopreserving the infusion bag comprising the harvested TIL population from step (f) using a cryopreservation process; and

(h) administering a therapeutically effective dosage of the third population of TILs from the infusion bag in step (g) to the subject.

82. The method of Claim 81, wherein the tumor sample is derived from a multilesional sampling method.

83. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

84. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody.

85. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent.

86. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated with a chemotherapeutic agent.

87. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has been previously treated a chemotherapeutic agent.

88. The method of Claims 85 to 87, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent.

89. The method of Claim 81, wherein the refractory NSCLC has low expression of PD-L1.

90. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

91. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has low expression of PD-L1.

92. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has low expression of PD-L1.

93. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has low expression of PD-L1

94. The method of Claim 81, wherein the refractory NSCLC has not been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

95. The method of Claim 81, wherein the refractory NSCLC has been previously treated with an anti-PD-1 and/or anti-PD-L1 antibody and has bulky disease at baseline.

96. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent and has bulky disease at baseline.

97. The method of Claim 81, wherein the refractory NSCLC has been treated with a chemotherapeutic agent but is not being currently treated with a chemotherapeutic agent and has bulky disease at baseline.

98. The method of Claims 94 to 97, wherein bulky disease is indicated where the maximal tumor diameter is greater than 7 cm measured in either the transverse or coronal plane or swollen lymph nodes with a short-axis diameter of 20 mm or greater.

99. The method of Claim 81, wherein the refractory NSCLC is refractory to at least two prior systemic treatment courses, not including neo-adjuvant or adjuvant therapies.

100. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-PD-1 or an anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

101. The method of Claim 81, wherein the refractory NSCLC is refractory to pembrolizumab or a biosimilar thereof.

102. The method of Claim 81, wherein the refractory NSCLC is refractory to nivolumab or a biosimilar thereof.

103. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof.

104. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and pembrolizumab or a biosimilar thereof.

105. The method of Claim 81, wherein the refractory NSCLC is refractory to an anti-CLTA-4 antibody, such as ipilimumab or a biosimilar thereof and nivolumab or a biosimilar thereof.

106. The method of Claim 81, wherein the refractory NSCLC is refractory to durvalumab or a biosimilar thereof.

107. The method of Claim 81, wherein the refractory NSCLC is refractory to atezolizumab or a biosimilar thereof.

108. The method of Claim 81, wherein the refractory NSCLC is refractory to avelumab or a biosimilar thereof.

109. The method of any one of Claims 81 to 108, wherein the initial expansion is

performed over a period of 21 days or less.

110. The method of any one of Claims 81 to 109, wherein the initial expansion is performed over a period of 14 days or less.

111. The method of any one of Claims 81 to 110, wherein the IL-2 is present at an initial concentration of between 1000 IU/mL and 6000 IU/mL in the first cell culture medium.

112. The method of any one of Claims 81 to 111, wherein 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 in the second cell culture medium.

113. The method of any one of Claims 81 to 112, wherein the initial expansion is performed using a gas permeable container.

114. The method of any one of Claims 81 to 113, wherein the rapid expansion is performed using a gas permeable container.

115. The method of any one of Claims 81 to 114, wherein 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.

116. The method of any one of Claims 81 to 115, wherein the second cell culture medium further comprises a cytokine selected from the group consisting of IL-4, IL-7, IL-15, IL-21, and combinations thereof.

117. The method of any one of Claims 81 to 116, further comprising the step of treating the patient with a non-myeloablative lymphodepletion regimen prior to administering the third population of TILs to the patient.

118. The method of Claim 117, wherein the non-myeloablative lymphodepletion regimen comprises the steps of administration of cyclophosphamide at a dose of 60 mg/m^2/day for two days followed by administration of fludarabine at a dose of 25 mg/m^2/day for five days.

119. The method of any one of Claims 81 to 118, further comprising the step of treating the patient with an IL-2 regimen starting on the day after administration of the third population of TILs to the patient.

120. The method of Claim 119, wherein the IL-2 regimen is a high-dose IL-2 regimen comprising 600,000 or 720,000 IU/kg of aldesleukin, or a biosimilar or variant thereof,

administered as a 15-minute bolus intravenous infusion every eight hours until tolerance.

121. The method according to any of the preceding Claims, wherein the NSCLC is refractory to a combination treatment or therapy comprising an anti-PD-1 and a chemotherapeutic agent.

122. The method of Claim 121, wherein the anti-PD-1 or the anti-PD-L1 antibody is selected from the group consisting of nivolumab, pembrolizumab, JS001, TSR-042, pidilizumab, (BGB-A317, SHR-1210, REGN2810, MDX-1106, PDR001, anti-PD-1 from clone: RMP1-14, an anti-PD-1 antibodies disclosed in U.S. Patent No. 8,008,449, durvalumab, atezolizumab, avelumab, and fragments, derivatives, variants, as well as biosimilars thereof.

123. The method according Claim 121, wherein the antiPD-1 is pembrolizumab.

124. The method according any one of Claims 121 to 123, wherein the chemotherapeutic agent is a platinum doublet chemotherapeutic agent(s).

125. The method according any one of Claims 121 to 123, wherein the platinum doublet therapy comprises:

i) a first chemotherapeutic agent selected from the group consisting of cisplatin and carboplatin,

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

126. The method according any one of Claims 121 to 125, wherein the chemotherapeutic agent(s) is in combination with pemetrexed.

127. The method according any one of Claims 121 to 126, wherein the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, and cisplatin.

128. The method according any one of Claims 121 to 127, wherein the NSCLC is refractory to a combination therapy comprising carboplatin, paclitaxel, pemetrexed, cisplatin, nivolumab, and ipilimumab.

Figure 1

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 F**

Final Formulation and/or Transfer to Infusion Bag

(optionally cryopreserve)

Figure 2A

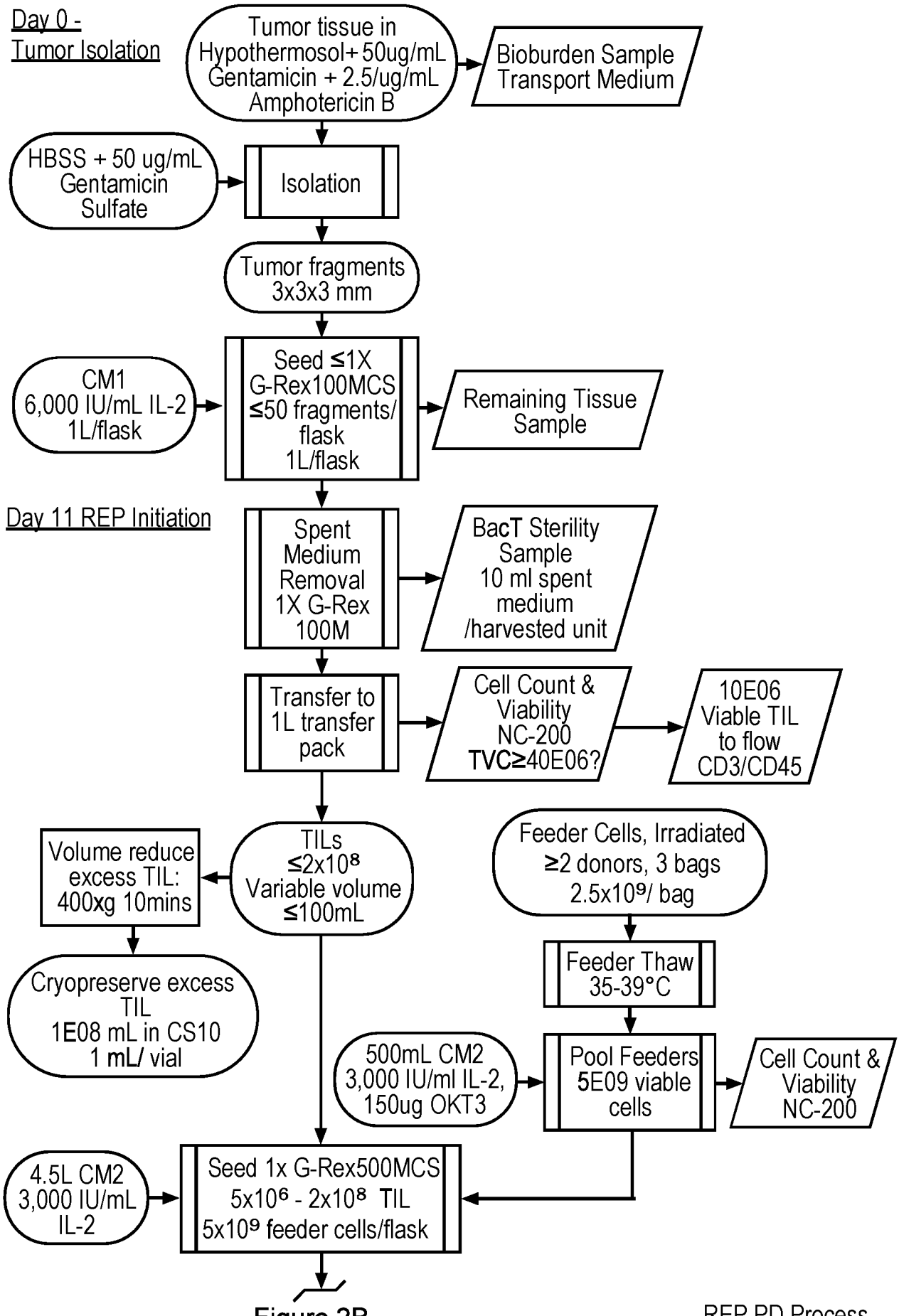


Figure 2B

REP PD Process
Page 1/3

Figure 2A

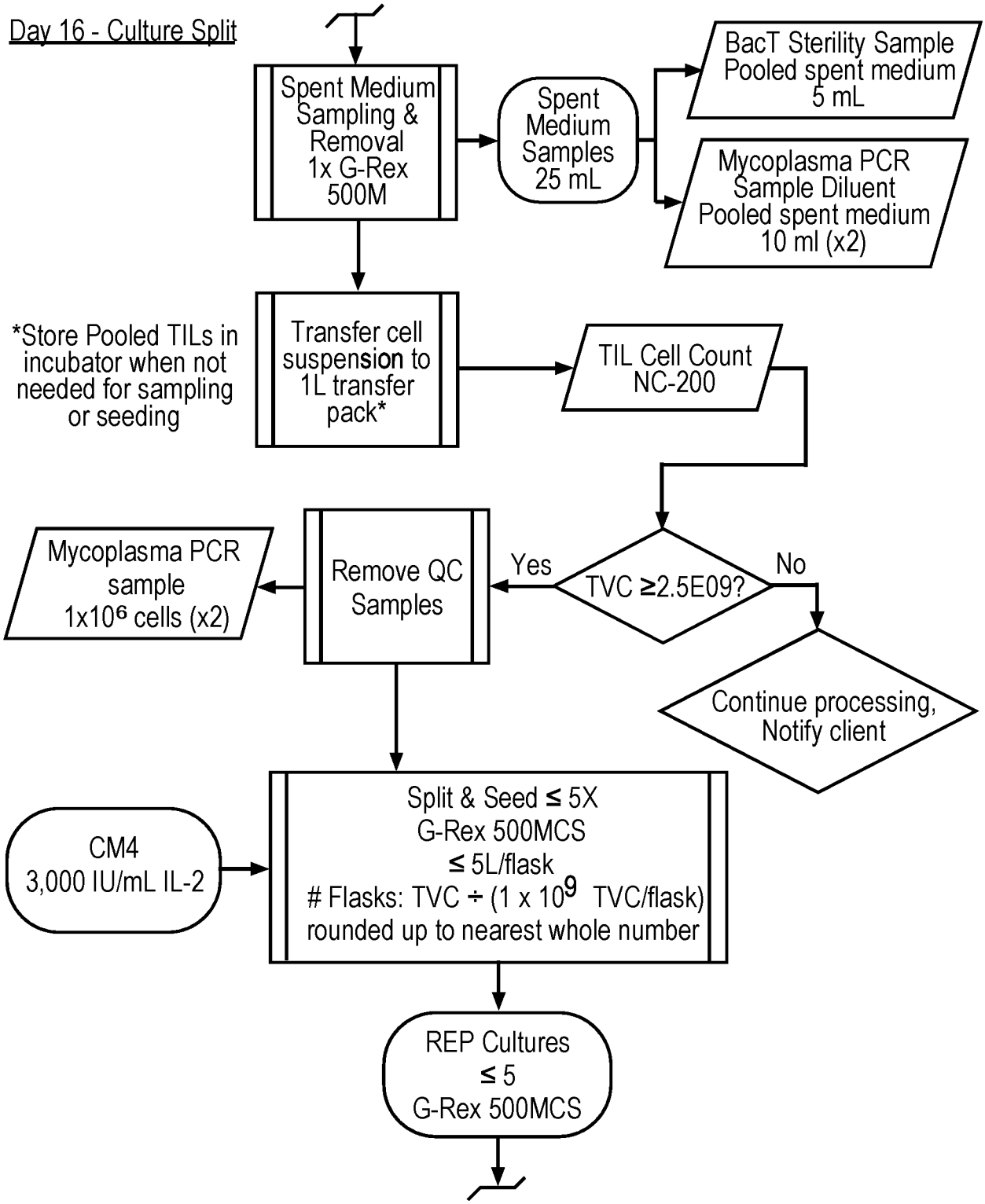


Figure 2C

Figure 2C

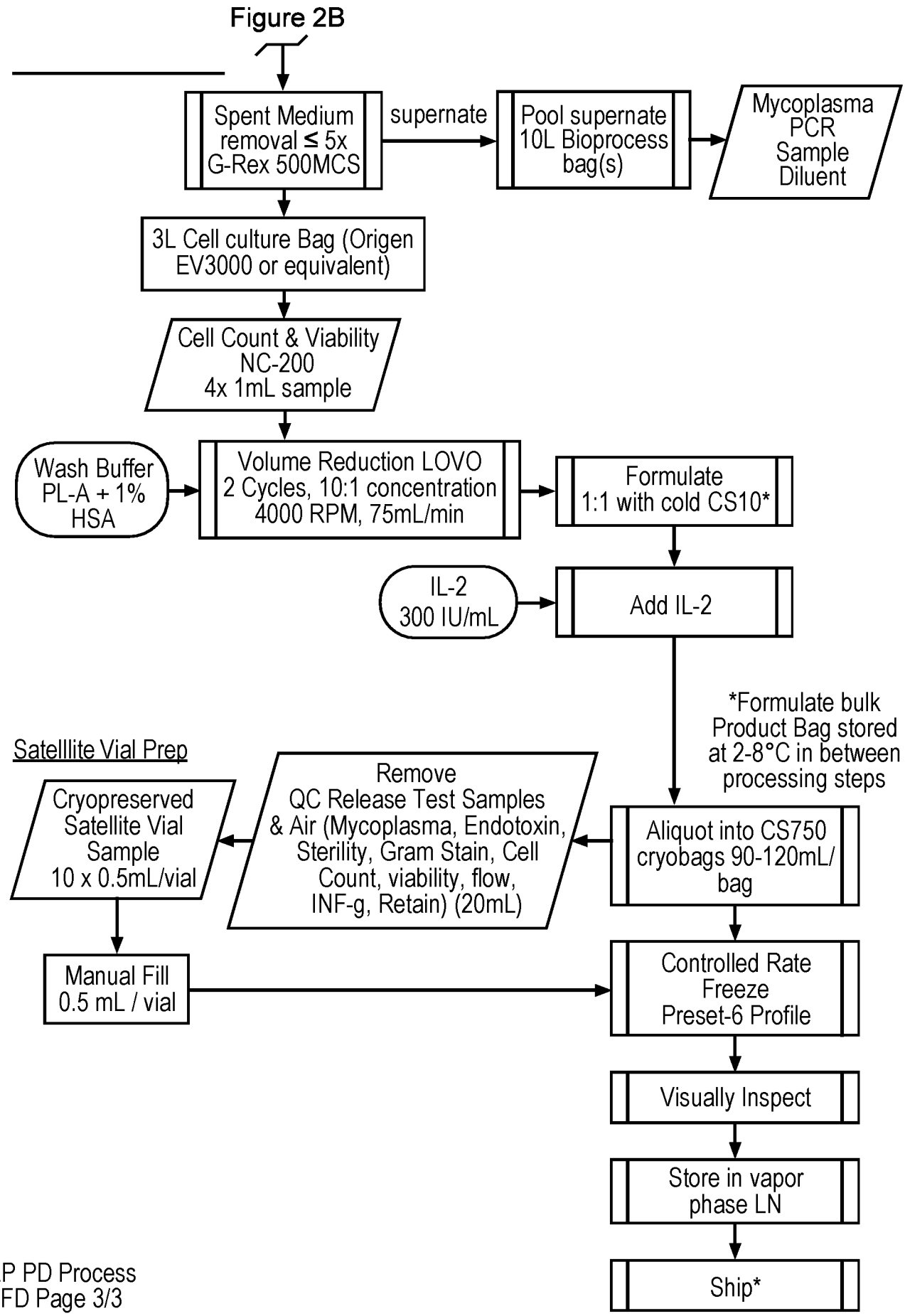


Figure 2B
Spent Medium removal ≤ 5x G-Rex 500MCS
supernate
Pool supernate 10L Bioprocess bag(s)
Mycoplasma PCR Sample Diluent
3L Cell culture Bag (Origen EV3000 or equivalent)
Cell Count & Viability NC-200 4x 1mL sample
Wash Buffer PL-A + 1% HSA
Volume Reduction LOVO 2 Cycles, 10:1 concentration 4000 RPM, 75mL/min
Formulate 1:1 with cold CS10*
IL-2 300 IU/mL
Add IL-2
*Formulate bulk Product Bag stored at 2-8°C in between processing steps
Satelllite Vial Prep
Cryopreserved Satellite Vial Sample 10 x 0.5mL/vial
Remove QC Release Test Samples & Air (Mycoplasma, Endotoxin, Sterility, Gram Stain, Cell Count, viability, flow, INF-g, Retain) (20mL)
Aliquot into CS750 cryobags 90-120mL/ bag
Manual Fill 0.5 mL / vial
Controlled Rate Freeze Preset-6 Profile
Visually Inspect
Store in vapor phase LN
Ship*
REP PD Process
PFD Page 3/3

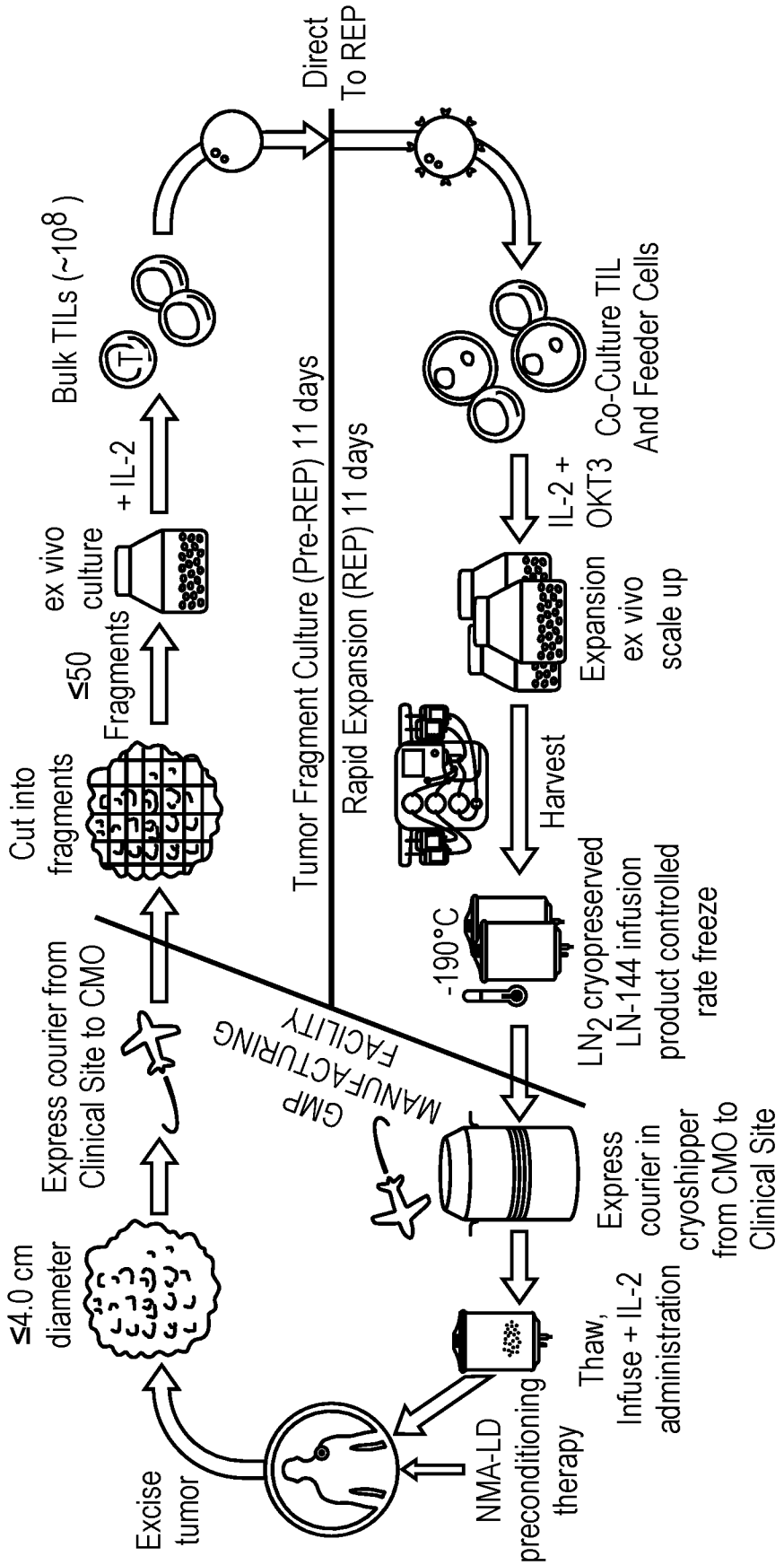

≤4.0 cm diameter
Excise tumor
Express courier from Clinical Site to CMO
Cut into fragments
≤50 Fragments
ex vivo culture
+ IL-2
Bulk TILs (~10^8)
Direct To REP
Tumor Fragment Culture (Pre-REP) 11 days
Rapid Expansion (REP) 11 days
GMP MANUFACTURING FACILITY
NMA-LD preconditioning therapy
Thaw, Infuse + IL-2 administration
Express courier in cryoshipper from CMO to Clinical Site
LN2 cryopreserved LN-144 infusion product controlled rate freeze
-190°C
Harvest
Expansion ex vivo scale up
IL-2 + OKT3
Co-Culture TIL And Feeder Cells

Figure 3

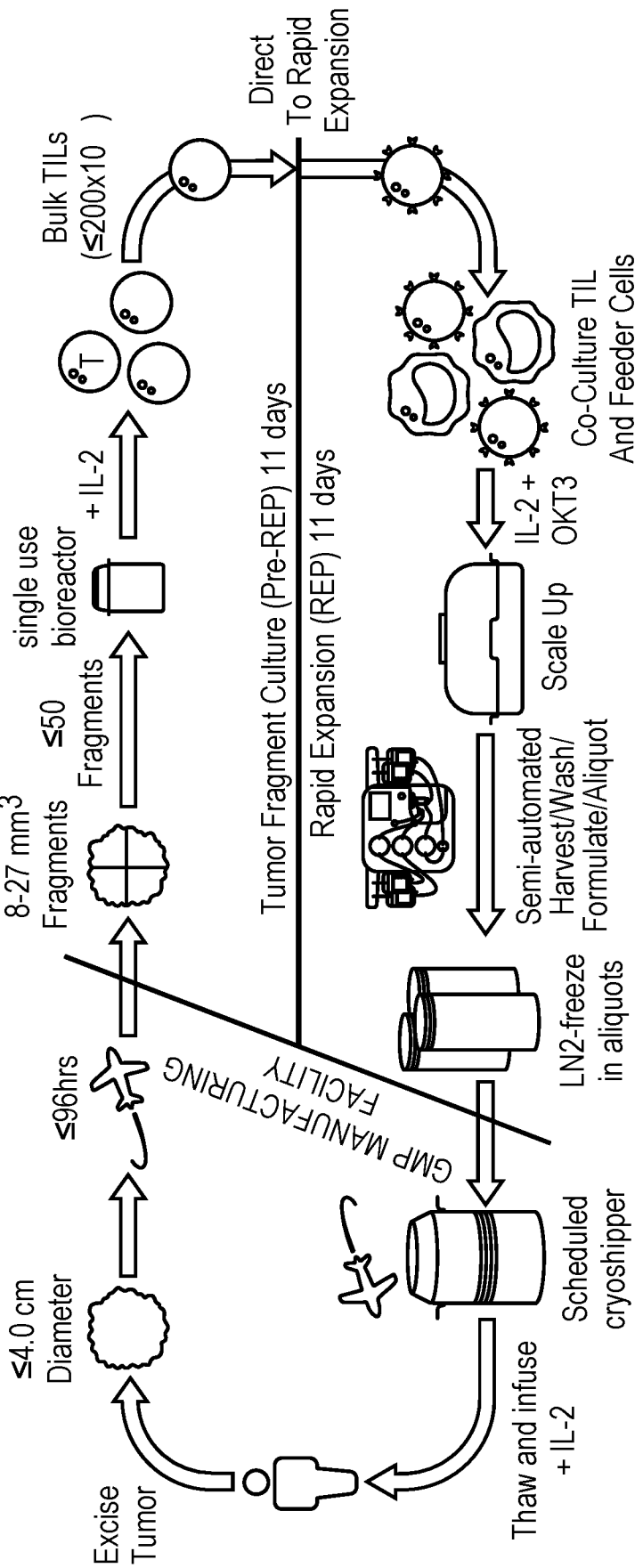

Excise Tumor
≤4.0 cm Diameter
≤96hrs
8-27 mm3 Fragments
≤50 Fragments
Closed single use bioreactor
+ IL-2
Bulk TILs (≤200x10)
Direct To Rapid Expansion
Tumor Fragment Culture (Pre-REP) 11 days
Rapid Expansion (REP) 11 days
GMP MANUFACTURING FACILITY
Co-Culture TIL And Feeder Cells
IL-2 + OKT3
Scale Up
Semi-automated Harvest/Wash/ Formulate/Aliquot
LN2-freeze in aliquots
Scheduled cryoshipper
Thaw and infuse + IL-2

Figure 4

Figure 5

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, and 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 6

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 >40×10^6 TIL	• Pre-REP TIL directly move to REP on day 11 • REP requires 25-200×10^6 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 • 5×10^6 TIL and 5×10^8 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 • 25-200×10^6 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	• Cyropreserved 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 7

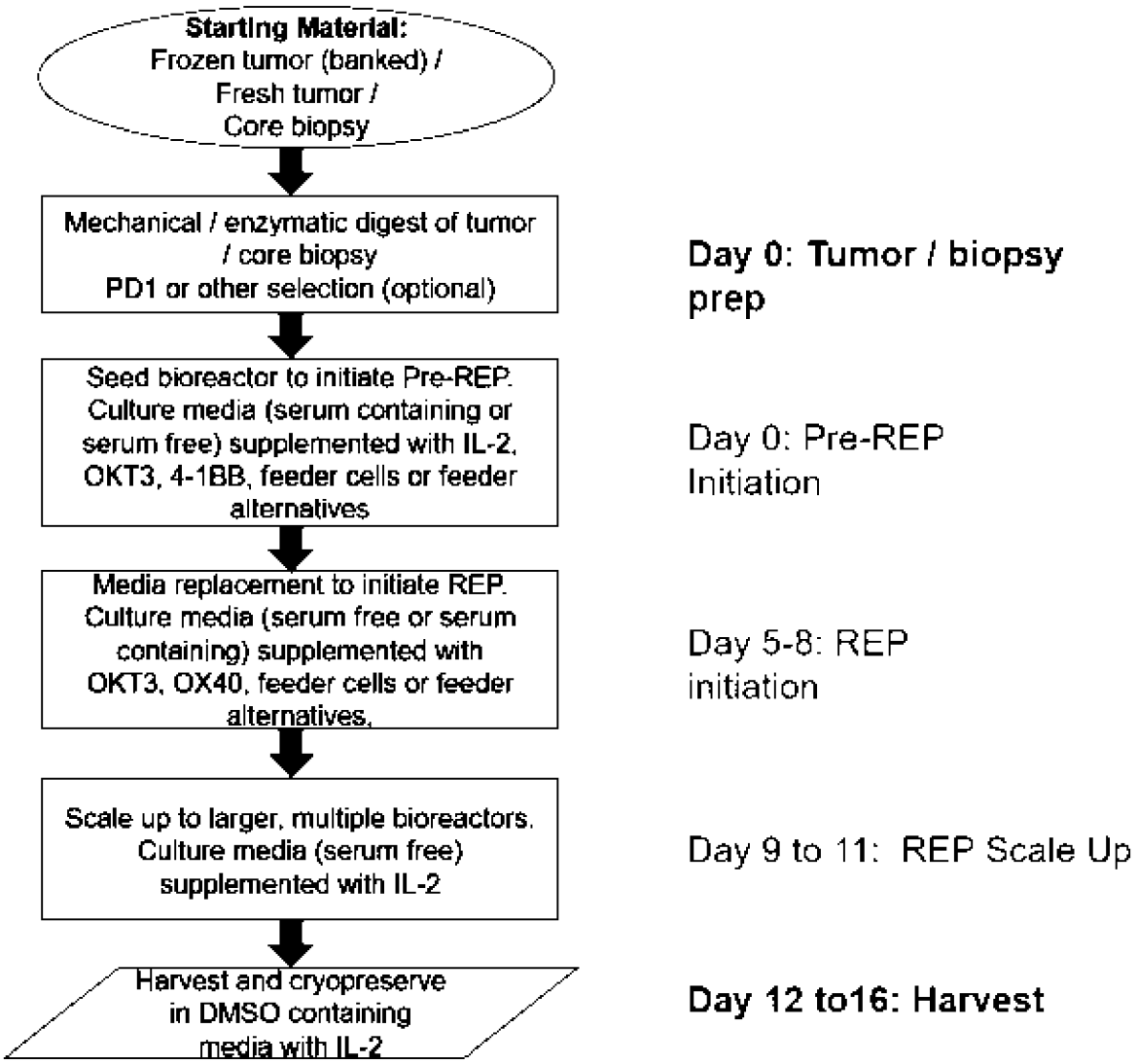

Starting Material:
Frozen tumor (banked) /
Fresh tumor /
Core biopsy
Mechanical / enzymatic digest of tumor / core biopsy
PD1 or other selection (optional)
Seed bioreactor to initiate Pre-REP. Culture media (serum containing or serum free) supplemented with IL-2, OKT3, 4-1BB, feeder cells or feeder alternatives
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 to16: Harvest

Figure 8A

Process 2A: 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 9

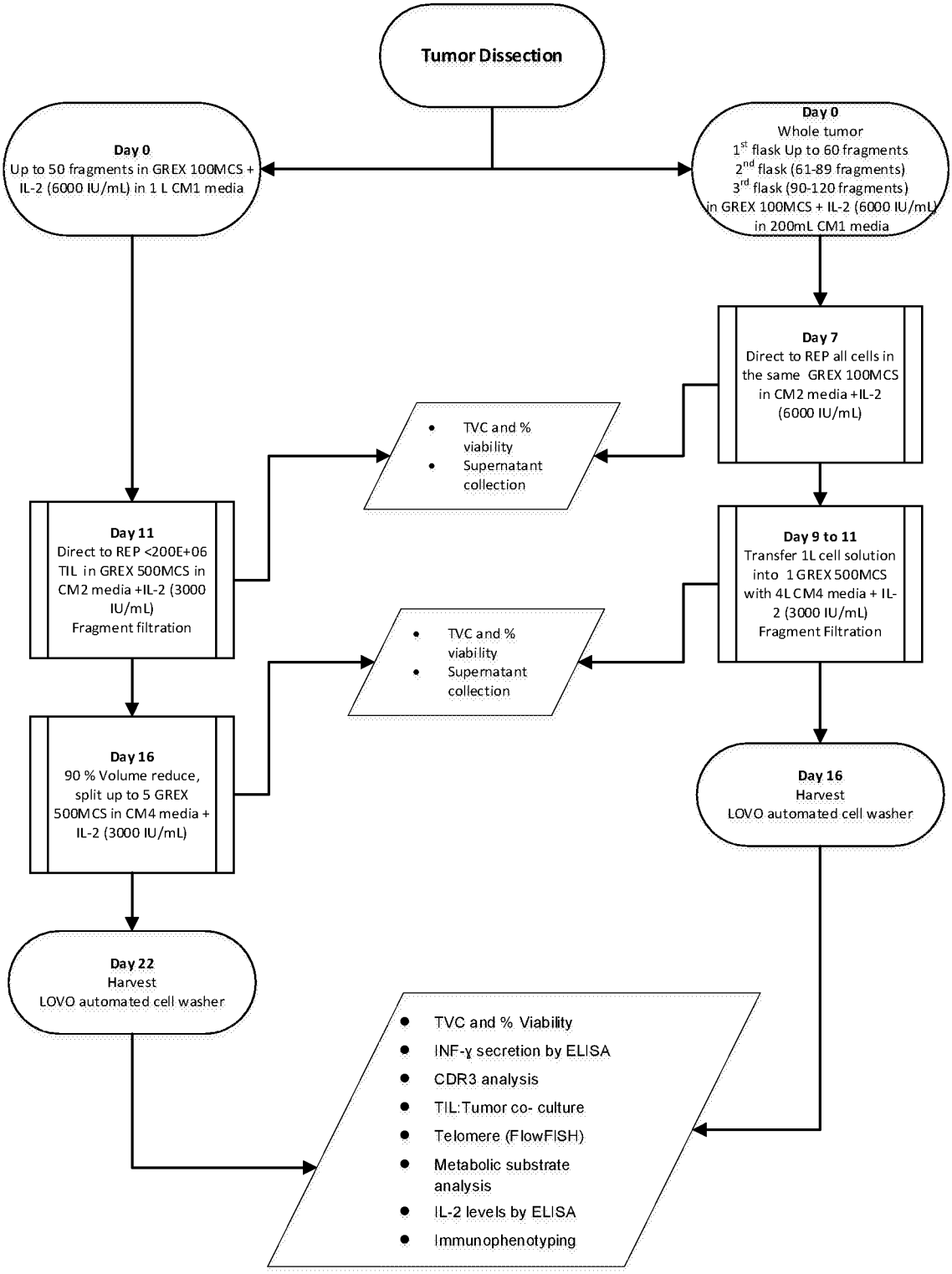
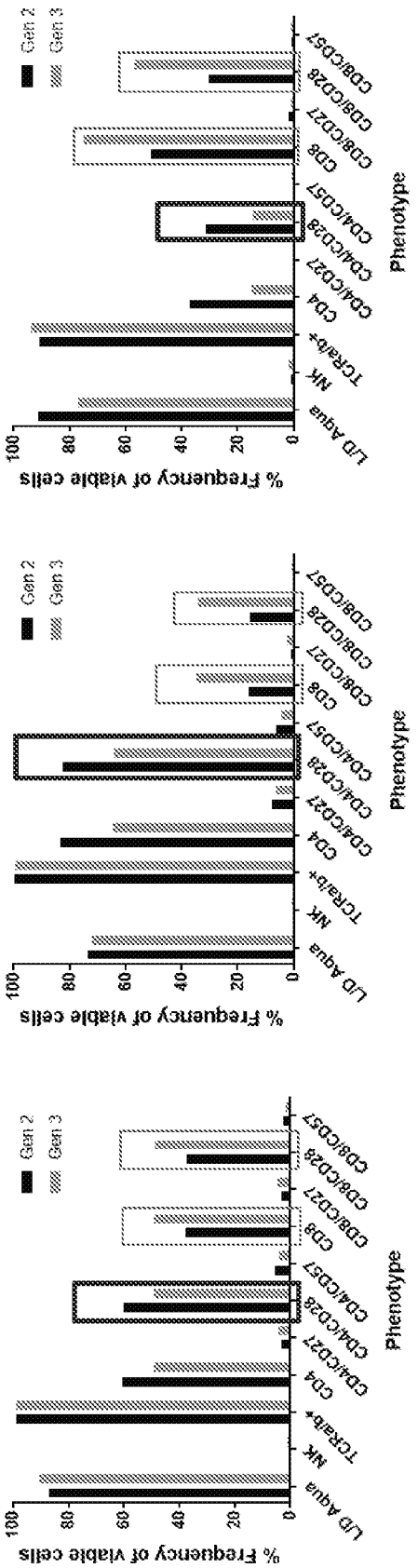
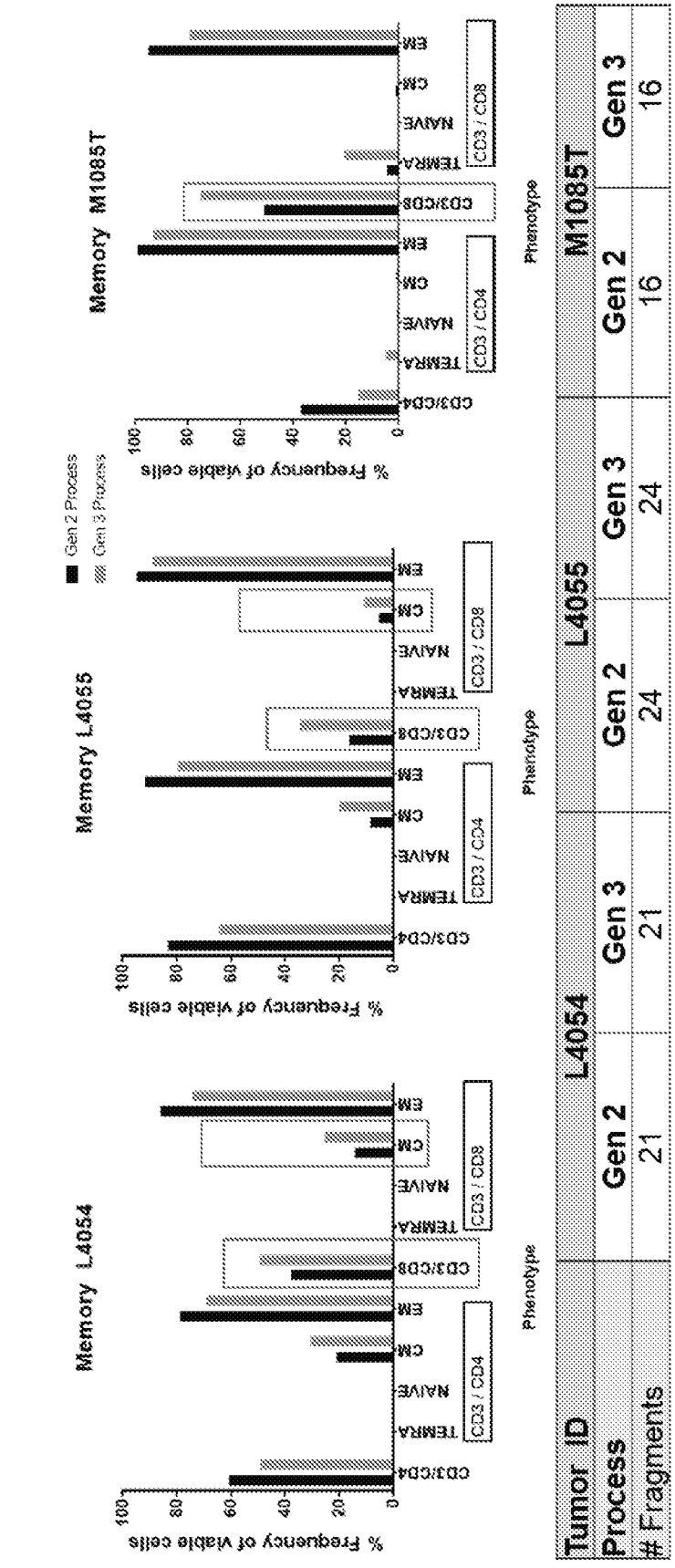

Tumor Dissection
Day 0
Up to 50 fragments in GREX 100MCS + IL-2 (6000 IU/mL) in 1 L CM1 media
Day 0
Whole tumor
1st flask Up to 60 fragments
2nd flask (61-89 fragments)
3rd flask (90-120 fragments)
in GREX 100MCS + IL-2 (6000 IU/mL) in 200mL CM1 media
Day 7
Direct to REP all cells in the same GREX 100MCS 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 10



Tumor ID	L4054		L4055		M1085T	
Process	Gen 2	Gen 3	Gen 2	Gen 3	Gen 2	Gen 3
# Fragments	21	21	24	24	16	16

Figure 11



Tumor ID	L4054		L4055		M1085T	
Process	Gen 2	Gen 3	Gen 2	Gen 3	Gen 2	Gen 3
# Fragments	21	21	24	24	16	16

Figure 12

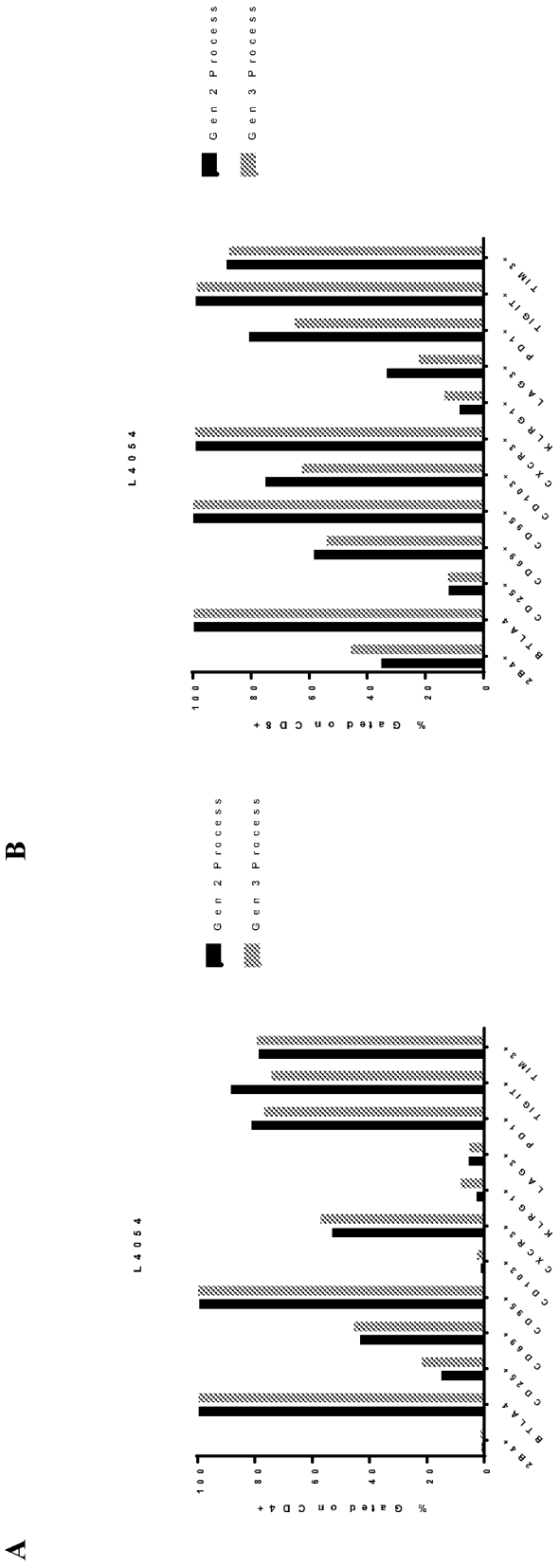

A
B
L4054
% Gated on CD4+
% Gated on CD8+
Gen 2 Process
Gen 3 Process
2B4+
BTLA4+
CD25+
CD69+
CD95+
CD103+
CXCR3+
KLRG1+
LAG3+
PD1+
TIGIT+
TIM3+
0
20
40
60
80
100

Figure 13

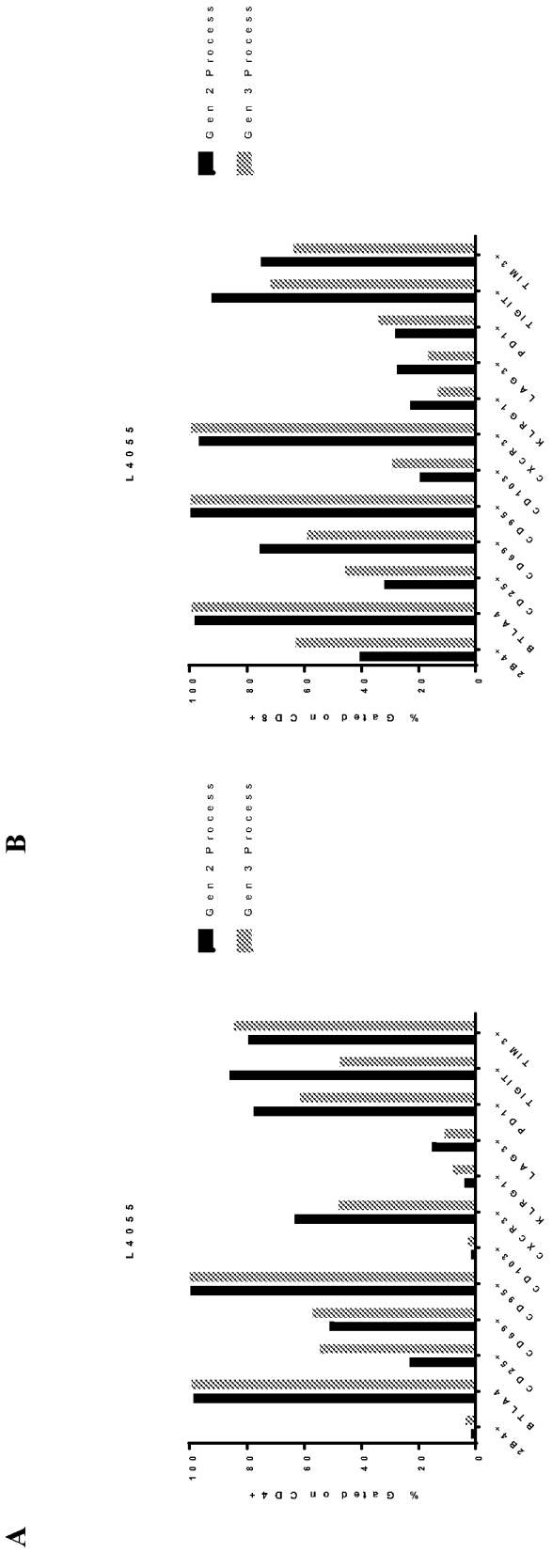

A
B
L4055
% Gated on CD4+
% Gated on CD8+
Gen 2 Process
Gen 3 Process
100
80
60
40
20
0
2B4+
BTLA4
CD25+
CD69+
CD95+
CD103+
CXCR3+
KLRG1+
LAG3+
PD1+
Tigit+
TIM3+

Figure 14

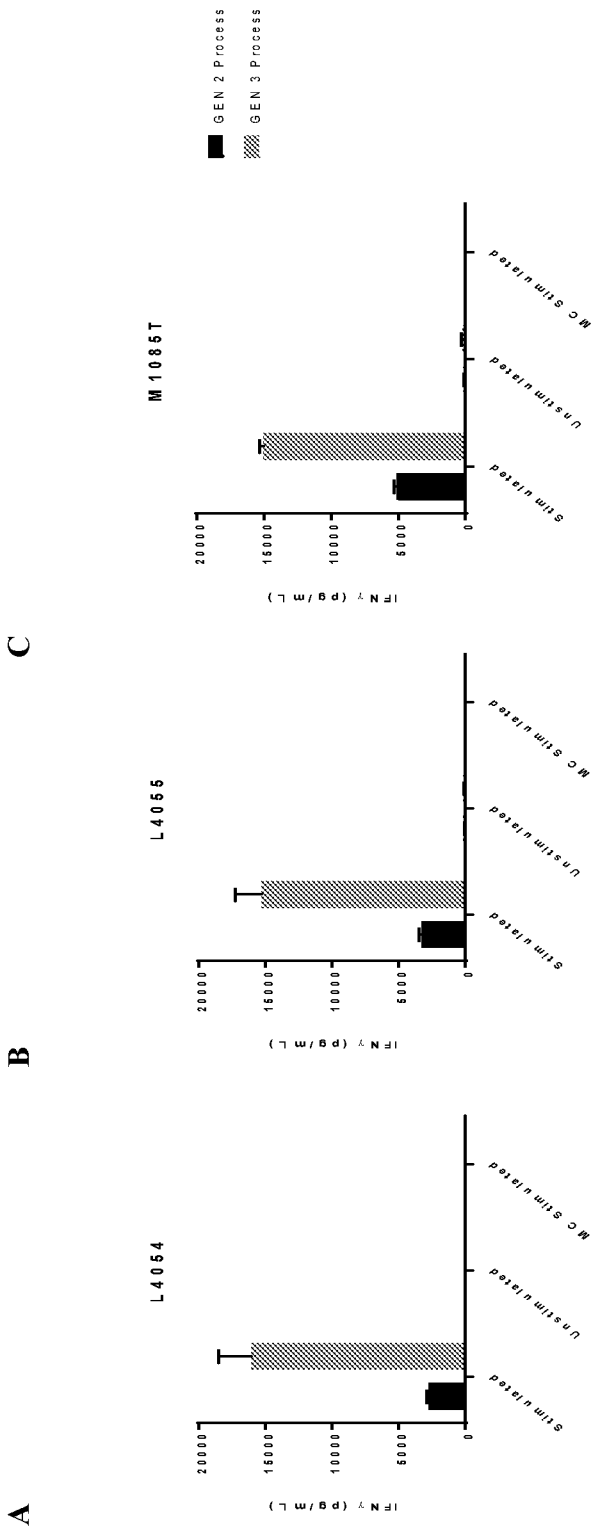

A
B
C
L4054
L4055
M1085T
GEN 2 Process
GEN 3 Process
IFNγ (pg/mL)
20000
15000
10000
5000
0
Stim u/tested
Unstim u/tested
MC Stim u/tested

Figure 15

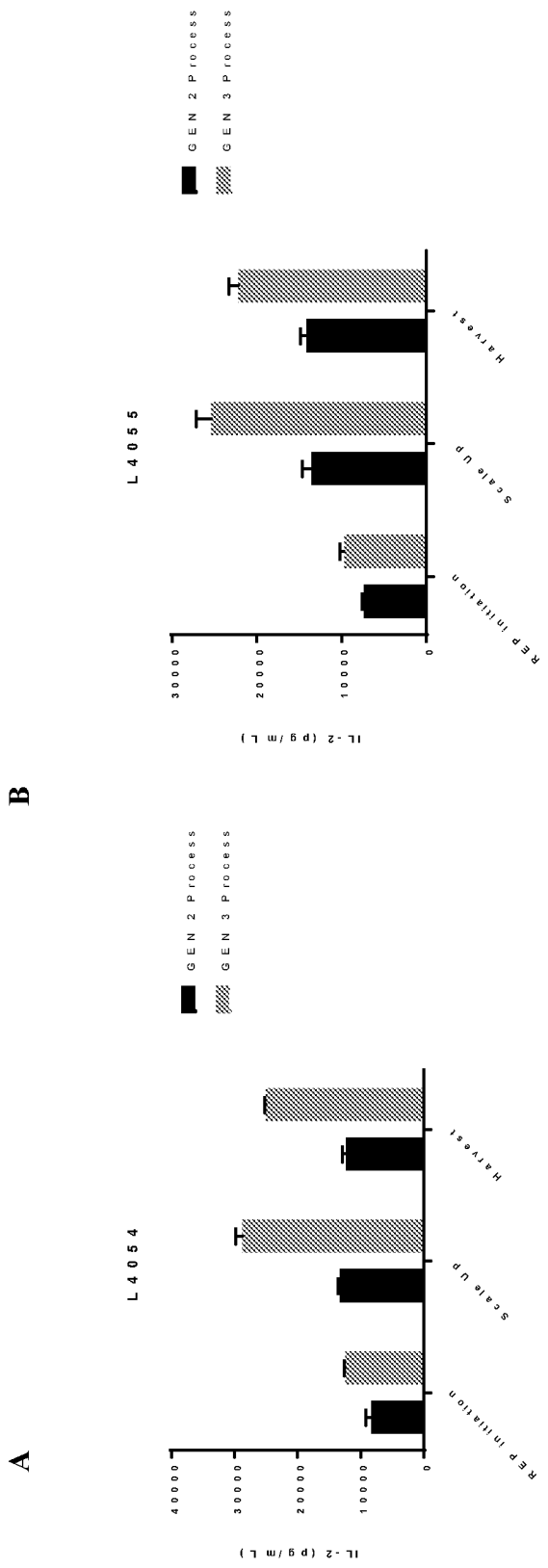

A
B
L4054
L4055
GEN 2 Process
GEN 3 Process
IL-2 (pg/mL)
40000
30000
20000
10000
0
Harvest
Scale up
Initiation

Figure 16

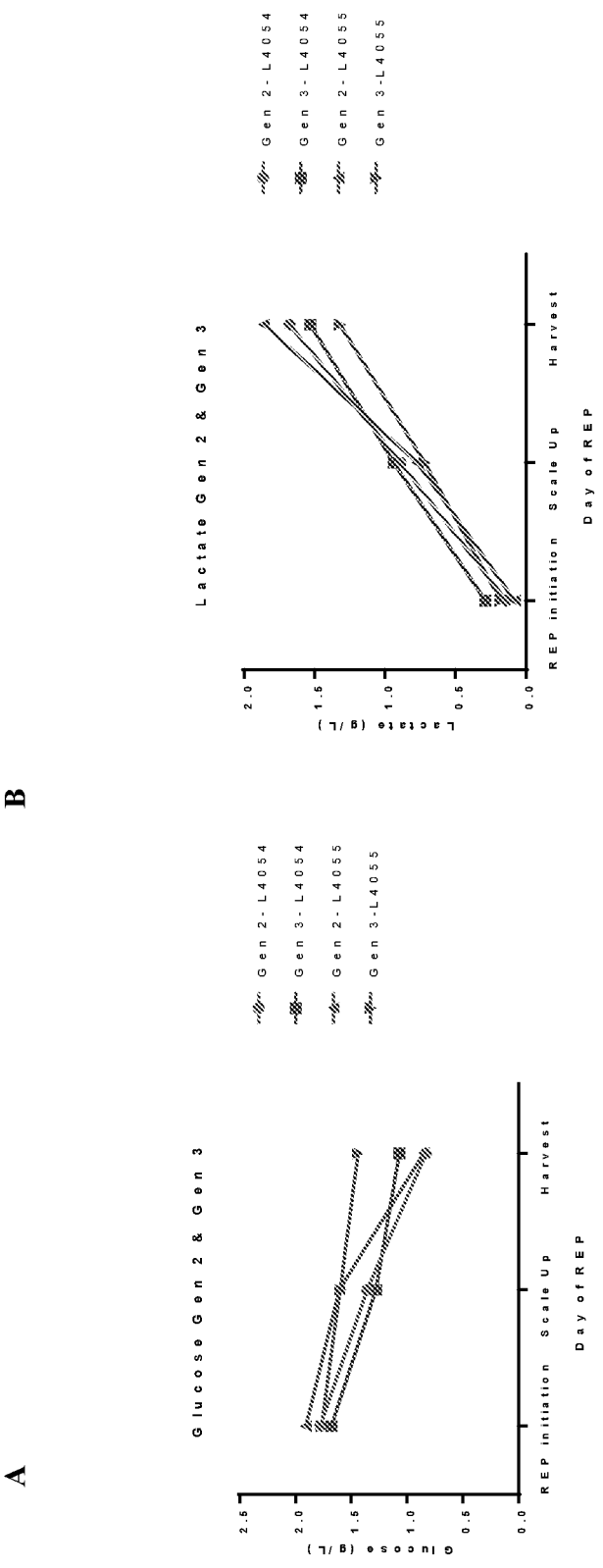
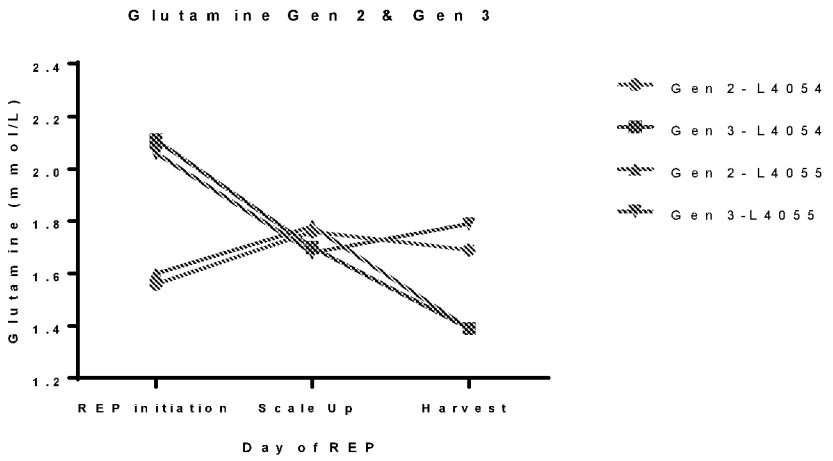

A
Glucose Gen 2 & Gen 3
Gen 2-L4054
Gen 3-L4054
Gen 2-L4055
Gen 3-L4055
Glucose (g/L)
2.5
2.0
1.5
1.0
0.5
0.0
REP initiation
Scale Up
Harvest
Day of REP
B
Lactate Gen 2 & Gen 3
Gen 2-L4054
Gen 3-L4054
Gen 2-L4055
Gen 3-L4055
Lactate (g/L)
2.0
1.5
1.0
0.5
0.0
REP initiation
Scale Up
Harvest
Day of REP

Figure 17

A


Glutamine Gen 2 & Gen 3
Glutamine (mmol/L)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
REP initiation
Scale Up
Harvest
Day of REP
Gen 2-L4054
Gen 3-L4054
Gen 2-L4055
Gen 3-L4055

B

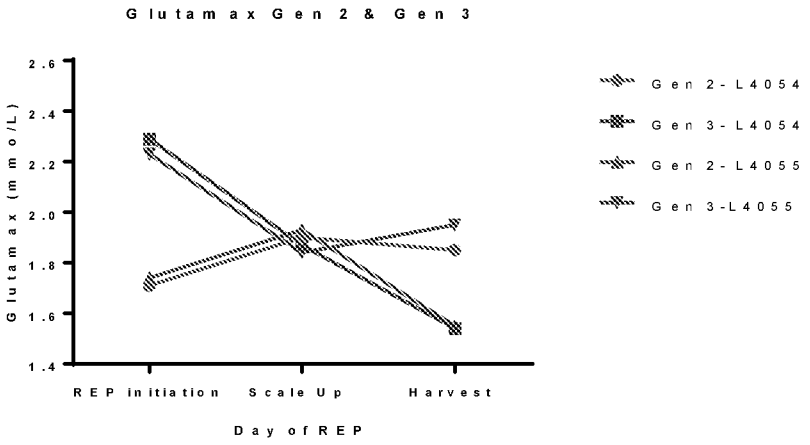

Glutamax Gen 2 & Gen 3
Glutamax (mmol/L)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
REP initiation
Scale Up
Harvest
Day of REP
Gen 2-L4054
Gen 3-L4054
Gen 2-L4055
Gen 3-L4055

Figure 17

C

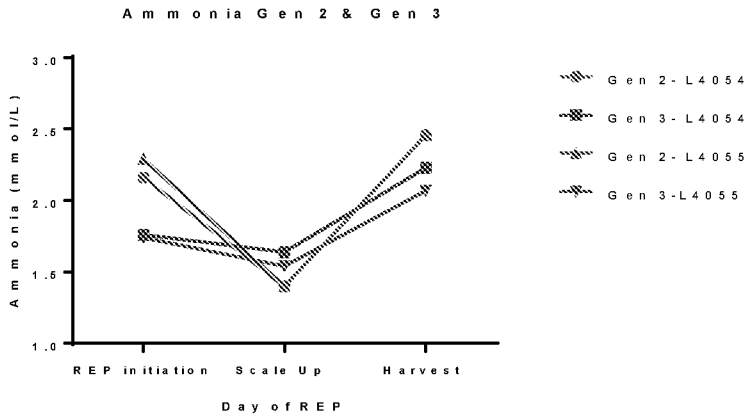

Ammonia Gen 2 & Gen 3
Ammonia (mmol/L)
3.0
2.5
2.0
1.5
1.0
REP initiation
Scale Up
Harvest
Day of REP
Gen 2-L4054
Gen 3-L4054
Gen 2-L4055
Gen 3-L4055

Figure 18

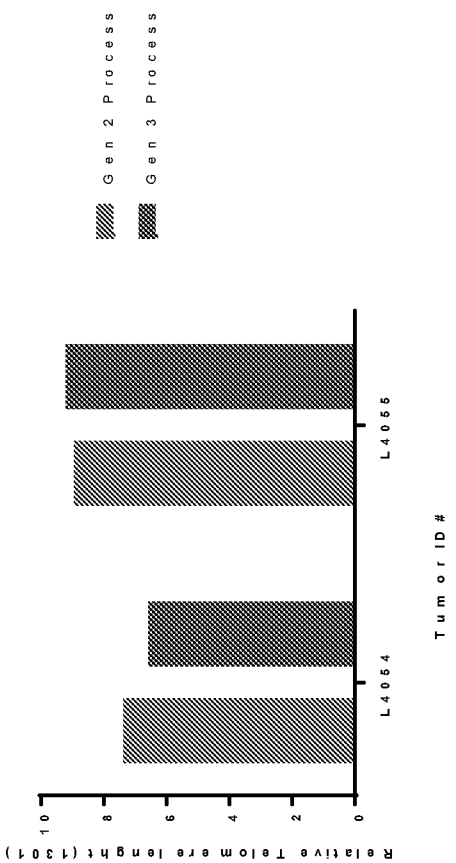

Gen 2 Process
Gen 3 Process
Relative Telomere length (1301)
0
2
4
6
8
10
L4054
L4055
Tumor ID#

Figure 19

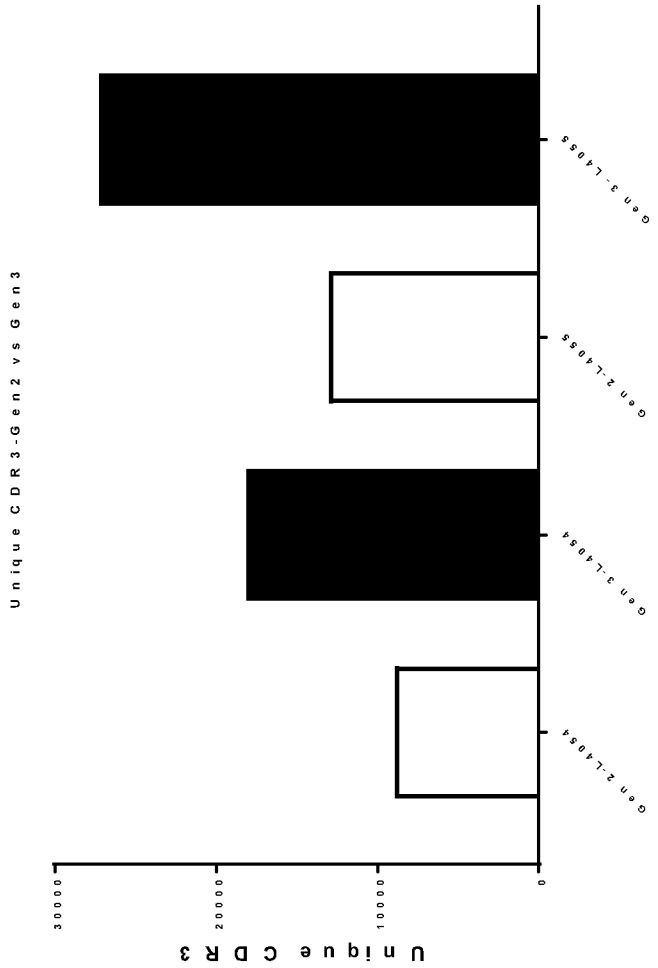

Unique CDR3-Gen2 vs Gen3
Unique CDR3
30000
20000
10000
0
Gen2-L4054
Gen3-L4054
Gen2-L4055
Gen3-L4055

Figure 20

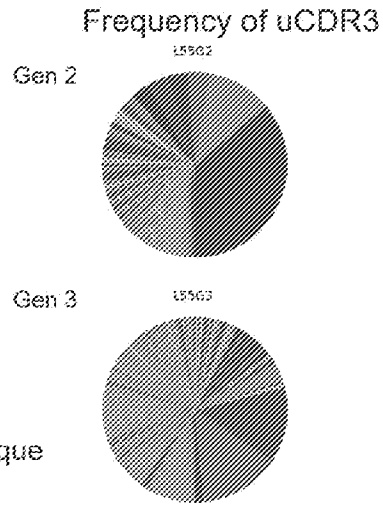
Frequency of uCDR3

# uCDR3 (% Overlap)	All uCDR3's		Top 80% uCDR3's	
	Gen 2	Gen 3	Gen 2	Gen 3
Gen 2-L4054	8915	4355 (48.85%)	205	199 (97.07%)
Gen 3-L4054	-	18130	-	223

* 199 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 97.07% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

Figure 21

# uCDR3 (% Overlap)	All uCDR3's		Top 80% uCDR3's	
	Gen 2	Gen 3	Gen 2	Gen 3
Gen 2-L4055	12996	6599 (50.77%)	1843	1833 (99.45%)
Gen 3-L4055	-	27246	-	2616



- 1833 sequences are shared between Gen 3 and Gen 2 final product, corresponding to 99.45% of top 80% of unique CDR3 sequences from Gen 2 shared with Gen 3 final product.

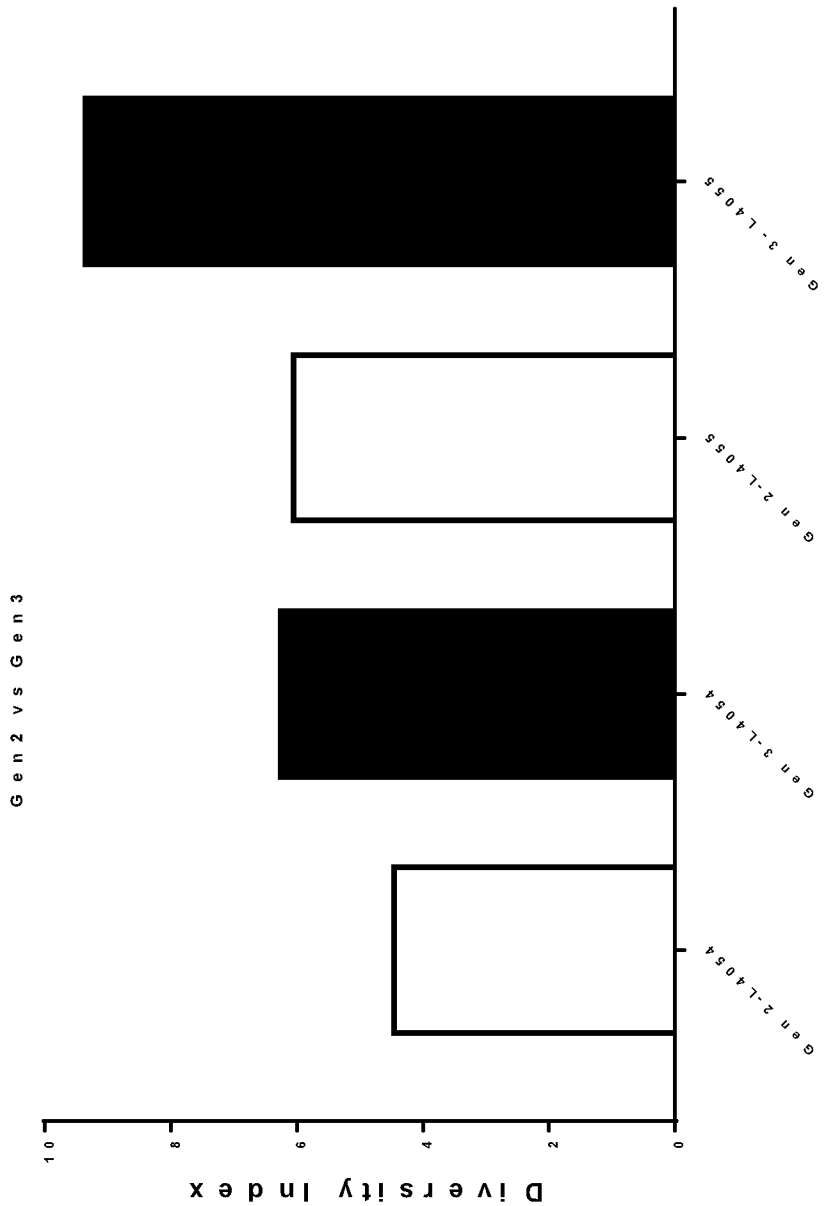

Gen2 vs Gen3
Diversity Index
0
2
4
6
8
10
Gen 2-L4054
Gen 3-L4054
Gen 2-L4055
Gen 3-L4055

Figure 22

Figure 23

Cell counts Day 7-Gen 3 REP initiation

Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
188	Total	2.46E+05	2.28E+05	2.31E+05	2.35E+05	4.42E+07
	Live	2.37E+05	2.27E+05	2.26E+05	2.30E+05	4.32E+07
	Dead	9.26E+03	7.03E+02	4.63E+03	4.86E+03	9.14E+05
	% Viability	96.20%	99.70%	98.00%	97.97%	
188	Total	8.26E+04	8.00E+04	7.66E+04	7.97E+04	1.50E+07
	Live	7.60E+04	7.54E+04	6.89E+04	7.34E+04	1.38E+07
	Dead	6.61E+03	4.63E+03	7.73E+03	6.32E+03	1.19E+06
	% Viability	92.00%	94.20%	89.90%	92.03%	
200	Total	2.44E+04	4.20E+04	1.05E+04	2.56E+04	5.13E+06
	Live	1.74E+04	2.80E+04	7.03E+03	1.75E+04	3.50E+06
	Dead	7.03E+03	1.40E+04	3.48E+03	8.17E+03	1.63E+06
	% Viability	71.20%	66.70%	66.90%	68.27%	

Figure 24

[0001] *Cell counts Day 11-Gen 2 REP initiation and Gen 3 Scale Up*

ID	Volume (mL)		Count 1 (cells/mL)	Count 3 (cells/mL)	Count 2 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3 #L4054 First Round	1000	Total	9.11E+05	1.01E+06	1.08E+06	1.00E+06	1.00E+09
		Live	8.60E+05	9.34E+05	1.01E+06	9.35E+05	9.35E+08
		Dead	5.09E+04	7.49E+04	6.74E+04	6.44E+04	6.44E+07
		% Viability	94.40%	92.60%	93.70%	93.57%	
Gen 3 #L4055 Second Round	1000	Total	8.56E+05	9.04E+05	8.81E+05	8.80E+05	8.80E+08
		Live	8.24E+05	8.67E+05	8.42E+05	8.44E+05	8.44E+08
		Dead	3.17E+04	3.70E+04	3.87E+04	3.58E+04	3.58E+07
		% Viability	96.30%	95.90%	95.60%	95.93%	
Gen 3 #M1085T	200	Total	2.32E+06	2.25E+06		2.29E+06	4.57E+08
		Live	1.68E+06	1.57E+06		1.63E+06	3.25E+08
		Dead	6.41E+05	6.79E+05		6.60E+05	1.32E+08
		% Viability	72.40%	69.90%		71.15%	
Gen 2 #L4054 First Round	142	Total	1.08E+06	9.84E+05	1.00E+06	1.02E+06	1.45E+08
		Live	1.06E+06	9.71E+05	9.79E+05	1.00E+06	1.42E+08
		Dead	2.05E+04	1.26E+04	2.12E+04	1.81E+04	2.57E+06
		% Viability	98.10%	98.70%	97.90%	98.23%	
Gen 2 #L4055 Second Round	96	Total	2.93E+05	3.05E+05	2.64E+05	2.87E+05	2.76E+07
		Live	2.88E+05	2.96E+05	2.55E+05	2.80E+05	2.68E+07
		Dead	4.72E+03	9.14E+03	8.26E+03	7.37E+03	7.08E+05
		% Viability	98.40%	97.00%	96.90%	97.43%	
Gen 2 #M1085T	200	Total	9.10E+04	5.60E+04		7.35E+04	1.47E+07
		Live	8.05E+04	4.21E+04		6.13E+04	1.23E+07
		Dead	1.04E+04	1.39E+04		1.22E+04	2.43E+06
		% Viability	88.50%	75.20%		81.85%	

Figure 25

[0002] *Cell counts Day 16-Gen 2 Scale Up and Gen 3 Harvest*

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3 #L4054 First Round -pre LOVO	1243.1	Total	1.40E+07	1.46E+07		1.43E+07	1.78E+10
		Live	1.32E+07	1.35E+07		1.34E+07	1.66E+10
		Dead	8.47E+05	1.12E+06		9.84E+05	1.22E+09
		% Viability	94.00%	92.40%		93.20%	
Gen 3 #L4054 First Round -post LOVO	330	Total	6.35E+07	6.07E+07		6.21E+07	2.05E+10
		Live	5.72E+07	5.44E+07		5.58E+07	1.84E+10
		Dead	6.35E+06	6.27E+06		6.31E+06	2.08E+09
		% Viability	90.00%	89.70%		89.85%	

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3 #L4055 Second Round -pre LOVO	1032.6	Total	9.49E+06	1.05E+07		1.00E+07	1.03E+10
		Live	8.90E+06	9.79E+06		9.35E+06	9.65E+09
		Dead	5.85E+05	6.75E+05		6.30E+05	6.51E+08
		% Viability	93.80%	93.60%		93.70%	
Gen 3 #L4055 Second Round -post LOVO	330	Total	3.31E+07	2.82E+07	3.18E+07	3.07E+07	1.01E+10
		Live	2.85E+07	2.46E+07	2.77E+07	2.66E+07	8.76E+09
		Dead	4.56E+06	3.61E+06	4.12E+06	4.09E+06	1.35E+09
		% Viability	86.20%	87.20%	87.10%	86.70%	

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3 #M1085T pre-LOVO	5000	Total	2.26E+06	2.64E+06		2.45E+06	1.23E+10
		Live	1.97E+06	2.31E+06		2.14E+06	1.07E+10
		Dead	2.85E+05	3.30E+05		3.08E+05	1.54E+09
		% Viability	87.40%	87.50%		87.45%	
Gen 3 #M1085T - post LOVO pre CS10 addition	150	Total	6.27E+07	5.44E+07		5.86E+07	8.78E+09
		Live	5.50E+07	4.74E+07		5.12E+07	7.68E+09
		Dead	7.70E+06	6.96E+06		7.33E+06	1.10E+09
		% Viability	87.70%	87.20%		87.45%	

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 2 #L4054 First Round	913	Total	3.53E+06	4.30E+06		3.92E+06	3.57E+09
		Live	3.35E+06	4.00E+06		3.68E+06	3.36E+09
		Dead	1.75E+05	3.03E+05		2.39E+05	2.18E+08
		% Viability	95.00%	93.00%		94.00%	
Gen 2 #L4055 Second Round	292	Total	1.29E+07	1.36E+07		1.33E+07	3.87E+09
		Live	1.16E+07	1.23E+07		1.20E+07	3.49E+09
		Dead	1.27E+06	1.23E+06		1.25E+06	3.65E+08
		% Viability	90.10%	90.90%		90.50%	
Gen 2 #M1085T	369	Total	6.75E+06	6.98E+06		6.87E+06	2.53E+09
		Live	5.22E+06	5.58E+06		5.40E+06	1.99E+09
		Dead	1.54E+06	1.40E+06		1.47E+06	5.42E+08
		% Viability	77.20%	79.90%		78.55%	

Figure 26

[0003] *Cell counts Day 22-Gen 2 Harvest*

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 2 #L4054 First Round -pre LOVO	1385.7	Total	3.55E+07	4.12E+07	4.03E+07	3.90E+07	5.40E+10
		Live	3.26E+07	3.73E+07	3.67E+07	3.55E+07	4.92E+10
		Dead	2.93E+06	3.87E+06	3.59E+06	3.46E+06	4.80E+09
		% Viability	91.70%	90.60%	91.10%	91.13%	
Gen 2 #L4054 First Round -post LOVO	330	Total	1.70E+08	1.79E+08	1.68E+08	1.72E+08	5.69E+10
		Live	1.49E+08	1.58E+08	1.48E+08	1.52E+08	5.01E+10
		Dead	2.16E+07	2.04E+07	2.00E+07	2.07E+07	6.82E+09
		% Viability	87.30%	88.60%	88.10%	87.95%	

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 2 #L4055 First Round -pre LOVO	1968.2	Total	3.15E+07	2.51E+07	2.97E+07	2.88E+07	5.66E+10
		Live	2.89E+07	2.25E+07	2.72E+07	2.62E+07	5.16E+10
		Dead	2.57E+06	2.61E+06	2.52E+06	2.57E+06	5.05E+09
		% Viability	91.80%	89.60%	91.50%	90.97%	
Gen 2 #L4055 First Round -post LOVO	330	Total	2.33E+08	1.89E+08	1.53E+08	1.92E+08	6.33E+10
		Live	2.03E+08	1.66E+08	1.33E+08	1.67E+08	5.52E+10
		Dead	3.00E+07	2.24E+07	1.94E+07	2.39E+07	7.90E+09
		% Viability	87.10%	88.10%	87.30%	87.50%	

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 2 #M1085T-pre LOVO	N/A	Total				#DIV/0!	#DIV/0!
		Live				#DIV/0!	#DIV/0!
		Dead				#DIV/0!	#DIV/0!
		% Viability				#DIV/0!	
Gen 2 #M1085T - post LOVO pre CS10 addition	150	Total	8.51E+07	9.05E+07		8.78E+07	1.32E+10
		Live	7.33E+07	7.79E+07		7.56E+07	1.13E+10
		Dead	1.18E+07	1.26E+07		1.22E+07	1.83E+09
		% Viability	86.10%	86.10%		86.10%	

For L4054 Gen 2, post LOVO count was extrapolated to 4 flasks, because was the total number of the study. 1 flask was contaminated, and the extrapolation was done for total = 6.67E+10

Figure 27

	S1/S2/All/Live | Freq. of Parent (%)	S1/S2/All/Live/CD14+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/CD3-/NK | Freq. of null (%)	S1/S2/All/Live/CD14-/CD19 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD4 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD4/CD27 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD4/CD28 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD4/CD56 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD4/CD57 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q1: CCR7-PE-A-, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q2: CCR7-PE-A+, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q3: CCR7-PE-A+, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q4: CCR7-PE-A-, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q5: CD62L-BV421-A-, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q6: CD62L-BV421-A+, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q7: CD62L-BV421-A+, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD4/Q8: CD62L-BV421-A-, CD45RA-AF700-A- | Freq. of Parent (%)
											TEMRA	NAÏVE	CM	EM	TEMRA	NAÏVE	CM	EM
A1 gen2 1st.fcs	87.2	1.77E-03	0.66	0	98.9	60.6	3.35	60.1	0.06	5.34	0.25	0.073	3.37	96.3	0.056	0.032	21.1	78.8
A2 gen2 2nd.fcs	73.5	0	0.12	0	99.7	83.1	7.94	82.6	0.14	6.18	0.16	0.047	2.39	97.4	0.036	0.014	8.3	91.6
A3 gen3 1st.fcs	90.4	0	0.97	1.72E-03	98.8	49.1	4.5	48.9	0.18	4.02	0.2	0.059	2.29	97.5	0.059	0.049	30.7	69.2
A4 gen3 2nd.fcs	72.1	2.56E-03	0.44	0.018	99.3	64.4	6.27	64	0.26	4.3	0.25	0.18	7.12	92.5	0.12	0.072	20.1	79.7
A5 PBMC.fcs	93.7	6.07	11.6	6.12	70.9	41.1	35.6	40.4	0.041	0.31	0.91	36.1	16.2	46.9	11.4	23.1	16.4	49.1
area was include to make the graph																		
TCRab+ are = CD3+																		

S1/S2/All/Live/CD14-/TCRab/CD8 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD8/CD27 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD8/CD28 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD8/CD56 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD8/CD57 | Freq. of null (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q1: CCR7-PE-A-, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q2: CCR7-PE-A+, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q3: CCR7-PE-A+, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q4: CCR7-PE-A-, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q5: CD62L-BV421-A-, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q6: CD62L-BV421-A+, CD45RA-AF700-A+ | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q7: CD62L-BV421-A+, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRab/CD8/Q8: CD62L-BV421-A-, CD45RA-AF700-A- | Freq. of Parent (%)	S1/S2/All/Live/CD14-/TCRgd | Freq. of null (%)
					TEMRA	NAÏVE	CM	EM	TEMRA	NAÏVE	CM	EM	
37.9	3.25	37.3	0.41	2.31	0.028	0	4.24	95.7	0.033	9.37E-03	14.1	85.9	0
16.1	1.08	15.8	0.67	0.17	0.042	0.014	4.76	95.2	0.071	0.014	5.24	94.7	0
49.2	4.83	48.5	0.71	1.53	0.056	6.97E-03	3.11	96.8	0.077	0.017	25.5	74.4	0
34.6	2.46	34	1.57	0.5	0.18	0.13	10.2	89.5	0.29	0.044	10.6	89.1	0
29.6	25.8	27.4	0.75	2.81	8.05	40.9	3.02	48	22.3	27.1	6.23	44.3	0

Figure 28

	CD3/CD4					
	CD38	HLA-DR	CCR7- CD45RA+	CCR7+CD45RA+	CCR7+ CD45RA-	CCR7-CD45RA-
GEN 3-Day 16-Harvest	42.4	95.2	4.96	0.7	1.22	93.1
GEN 2-D22-Harvest	50.4	98	0.61	0.13	0.3	99

CD3	CD3/CD8						
CD8	CD38	HLA-DR	CCR7-CD45RA+	CCR7+CD45RA+	CCR7+CD45RA-	CCR7-CD45RA-	
	77.6	83.2	90.3	20.5	0.013	0.06	79.5
	54.3	88	83.2	4.24	0.026	1.05	94.7

Figure 29

			CD4											
	CD3+	CD4+	2B4+	BTLA+	CD103+	CD25+	CD69+	CD95+	CXCR3+	KLRG1+	LAG3+	PD1+	TIGIT+	TIM3+
Gen 2-L4054			0.61	99.50	1.05	14.80	43.20	99.40	52.90	2.65	5.36	81.20	88.40	78.60
Gen 3-L4054			1.11	99.50	2.28	21.70	45.40	99.70	56.90	8.06	5.07	76.70	74.10	79.30
Gen 2-L4055			1.61	98.60	1.56	23.10	51.10	99.80	63.30	3.86	15.30	77.60	86.00	79.50
Gen 3- L4055			3.57	99.30	2.61	54.40	57.00	99.90	48.00	8.02	10.80	61.40	47.50	84.60

			CD8											
Gen 2-L4054		CD8+	2B4+	BTLA4+	CD103+	CD25+	CD95+	CD69+	CXCR3+	KLRG1+	LAG3+	PD1+	TIGIT+	TIM3+
Gen 3-L4054			35.20	99.50	74.90	11.80	99.80	58.30	99.10	8.03	33.10	80.60	99.10	88.30
Gen 2-L4055			45.50	99.60	62.40	12.10	99.90	54.00	99.30	13.40	22.10	64.90	98.50	87.50
Gen 3- L4055			40.60	98.20	19.50	32.00	99.70	75.50	96.80	22.80	27.40	28.10	92.30	75.10
			62.80	99.20	29.20	45.50	99.70	58.80	99.60	13.10	16.60	34.10	71.70	63.60

Figure 30

	1	2	3	4	5	6	7	8	9	10	11	12	
A	STD1	STD1	STD1	TIL- Gen 2 stimulated	TIL- Gen 2 stimulated	TIL- Gen 2 stimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	MC- Stimulated xG2	MC- Stimulated xG2	MC- Stimulated xG2	Well ID
	1000	1000	1000	3	3	3	3	3	3	3	3	3	Conc/Dil
B	STD2	STD2	STD2	TIL- Gen 2 stimulated	TIL- Gen 2 stimulated	TIL- Gen 2 stimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	MC- Stimulated xG2	MC- Stimulated xG2	MC- Stimulated xG2	Well ID
	500	500	500	9	9	9	9	9	9	9	9	9	Conc/Dil
C	STD3	STD3	STD3	TIL- Gen 2- stimulated	TIL- Gen 2- stimulated	TIL- Gen 2- stimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2- Unstimulated	MC- Unstimulated xG2	MC- Unstimulated xG2	MC- Unstimulated xG2	Well ID
	250	250	250	27	27	27	27	27	27	3	3	3	Conc/Dil
D	STD4	STD4	STD4	TIL- Gen 2- stimulated	TIL- Gen 2- stimulated	TIL- Gen 2- stimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	TIL- Gen 2 Unstimulated	MC- Unstimulated xG2	MC- Unstimulated xG2	MC- Unstimulated xG2	Well ID
	125	125	125	81	81	81	81	81	81	9	9	9	Conc/Dil
E	STD5	STD5	STD5	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	MC- Stimulated xG3	MC- Stimulated xG3	MC- Stimulated xG3	Well ID
	62.5	62.5	62.5	3	3	3	3	3	3	3	3	3	Conc/Dil
F	STD6	STD6	STD6	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	MC- Stimulated xG3	MC- Stimulated xG3	MC- Stimulated xG3	Well ID
	31.3	31.3	31.3	9	9	9	9	9	9	9	9	9	Conc/Dil
G	STD7	STD7	STD7	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	MC- Unstimulated xG3	MC- Unstimulated xG3	MC- Unstimulated xG3	Well ID
	15.6	15.6	15.6	27	27	27	27	27	27	3	3	3	Conc/Dil
H	STD8	STD8	STD8	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- stimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	TIL- Gen 3- Unstimulated	MC- Unstimulated xG3	MC- Unstimulated xG3	MC- Unstimulated xG3	Well ID
	0	0	0	81	81	81	81	81	81	9	9	9	Conc/Dil

22.6
22.6

	1	2	3	4	5	6	7	8	9	10	11	12	
A	2.866	2.882	2.886	2.797	2.164	2.918	0.117	0.116	0.117	0.105	0.108	0.102	450
	0.05	0.05	0.05	0.05	0.048	0.051	0.041	0.043	0.041	0.041	0.043	0.041	570
	2.816	2.832	2.836	2.746	2.117	2.867	0.076	0.074	0.076	0.064	0.065	0.06	Delta
	994.773	1002.343	1004.079	963.405	694.982	1018.375	1.825	1.421	1.952	<0.000	<0.000	<0.000	[Concentration]
				2890.216	2084.946	3055.126	5.474	4.262	5.855	<0.000	<0.000	<0.000	xdil factor
B	1.659	1.654	1.65	1.181	0.9	1.215	0.107	0.107	0.108	0.106	0.105	0.102	450
	0.046	0.046	0.046	0.045	0.047	0.045	0.042	0.041	0.042	0.043	0.042	0.042	570
	1.613	1.608	1.604	1.136	0.853	1.17	0.066	0.066	0.067	0.062	0.063	0.061	Delta
	499.866	497.826	496.528	329.765	235.273	341.438	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	[Concentration]
				2967.887	2117.46	3072.942	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	xdil factor
C	0.952	0.966	0.94	0.494	0.391	0.505	0.106	0.107	0.107	0.107	0.105	0.103	450
	0.044	0.048	0.043	0.042	0.042	0.043	0.041	0.042	0.042	0.043	0.042	0.042	570
	0.908	0.919	0.898	0.452	0.349	0.462	0.065	0.065	0.065	0.063	0.063	0.062	Delta
	253.244	256.8	249.399	109.334	78.83	112.411	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	[Concentration]
				2952.028	2128.412	3035.11	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	xdil factor
D	0.549	0.541	0.542	0.258	0.218	0.259	0.102	0.101	0.103	0.111	0.102	0.101	450
	0.044	0.044	0.043	0.042	0.041	0.047	0.041	0.042	0.042	0.05	0.042	0.043	570
	0.505	0.498	0.499	0.216	0.176	0.212	0.06	0.059	0.061	0.061	0.06	0.057	Delta
	125.458	123.174	123.508	40.262	29.15	39.101	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	[Concentration]
				3261.251	2361.138	3167.212	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	xdil factor
E	0.334	0.332	0.332	OVRFLW	OVRFLW	OVRFLW	0.114	0.11	0.113	0.103	0.102	0.104	450
	0.043	0.042	0.042	[illegible]	[illegible]	[illegible]	0.041	0.041	0.042	0.042	0.042	0.043	570
	0.291	0.29	0.29	?????	?????	?????	0.073	0.069	0.072	0.062	0.061	0.061	Delta
	61.826	61.537	61.594	?????	?????	?????	1.145	0.285	0.822	<0.000	<0.000	<0.000	[Concentration]
				?????	?????	?????	3.435	0.854	2.465	<0.000	<0.000	<0.000	xdil factor
F	0.222	0.222	0.224	[illegible]	3.893	[illegible]	0.114	0.104	0.107	0.101	0.103	0.101	450
	0.042	0.043	0.042	0.056	0.056	0.057	0.041	0.041	0.042	0.042	0.041	0.042	570
	0.18	0.179	0.181	[illegible]	3.838	[illegible]	0.073	0.063	0.066	0.059	0.062	0.059	Delta
	30.212	29.82	30.519	1474.838	>1500.000	1482.263	1.145	<0.000	<0.000	<0.000	<0.000	<0.000	[Concentration]
				13273.55	>13500.000	13340.37	10.304	<0.000	<0.000	<0.000	<0.000	<0.000	xdil factor
G	0.174	0.17	0.172	1.996	1.997	2.017	0.114	0.109	0.114	0.106	0.107	0.104	450
	0.041	0.041	0.041	0.048	0.047	0.049	0.042	0.042	0.041	0.042	0.041	0.042	570
	0.132	0.128	0.131	1.948	1.949	1.968	0.073	0.067	0.073	0.063	0.065	0.062	Delta
	16.911	15.874	16.447	627.703	628.253	635.61	1.12	<0.000	1.17	<0.000	<0.000	<0.000	[Concentration]
				16947.99	16962.84	17161.47	30.239	<0.000	31.588	<0.000	<0.000	<0.000	xdil factor
H	0.117	0.111	0.114	0.84	0.867	0.857	0.11	0.11	0.115	0.109	0.11	0.113	450
	0.045	0.043	0.044	0.044	0.044	0.044	0.041	0.042	0.043	0.042	0.043	0.042	570
	0.072	0.068	0.07	0.797	0.822	0.813	0.069	0.068	0.073	0.067	0.067	0.071	Delta
	0.822	0.073	0.405	216.931	225.269	222.13	0.119	0.05	1.145	<0.000	<0.000	0.748	[Concentration]
				17571.43	18246.77	17992.49	9.644	4.039	92.74	<0.000	<0.000	60.557	xdil factor

				Average	Average
Gen 2-Stimulated+TIL 1:3	2890.216	2084.946	3055.126	2676.76267	2757.81
Gen 2-Stimulated+TIL 1:9	2967.887	2117.46	3072.942	2719.42967	
Gen 2-Stimulated+TIL 1:27	2952.028	2128.412	3035.11	2705.18333	
Gen 2-Stimulated+TIL 1:81	3261.251	2361.138	3167.212	2929.867	
Gen 3-Stimulated+TIL 1:3	?????	?????	?????	#DIV/0!	#DIV/0!
Gen 3-Stimulated+TIL 1:9	13273.546	>13500.0(	13340.37	13306.9575	16089.32
Gen 3-Stimulated+TIL 1:27	16947.994	16962.84	17161.47	17024.1	
Gen 3-Stimulated+TIL 1:81	17571.427	18246.77	17992.49	17936.8973	
Gen 2-Unstimulated 1:3	5.474	4.262	5.855	5.197	5.20
Gen 2-Unstimulated 1:9	<0.000	<0.000	<0.000	#DIV/0!	
Gen 2-Unstimulated 1:27	<0.000	<0.000	<0.000	#DIV/0!	
Gen 2-Unstimulated 1:81	<0.000	<0.000	<0.000	#DIV/0!	
Gen 3-Unstimulated 1:3	3.435	0.854	2.465	2.25133333	19.74
Gen 3-Unstimulated 1:9	10.304	<0.000	<0.000	10.304	
Gen 3-Unstimulated 1:27	30.239	<0.000	31.588	30.9135	
Gen 3-Unstimulated 1:81	9.644	4.039	92.74	35.4743333	
Gen 2-Media Control Stimulated 1:3	<0.000	<0.000	<0.000	#DIV/0!	0.00
Gen 2-Media Control Stimulated 1:9	<0.000	<0.000	<0.000	#DIV/0!	
Gen 3-Media Control Stimulated 1:3	<0.000	<0.000	<0.000	#DIV/0!	0.00
Gen 3-Media Control Stimulated 1:9	<0.000	<0.000	<0.000	#DIV/0!	
Gen 2-Media Control Unstimulated 1:3	<0.000	<0.000	<0.000	#DIV/0!	0.00
Gen 2-Media Control Unstimulated 1:9	<0.000	<0.000	<0.000	#DIV/0!	
Gen 3-Media Control Unstimulated 1:3	<0.000	<0.000	<0.000	0	30.28
Gen 3-Media Control Unstimulated 1:9	<0.000	<0.000	60.557	60.557	

Figure 31

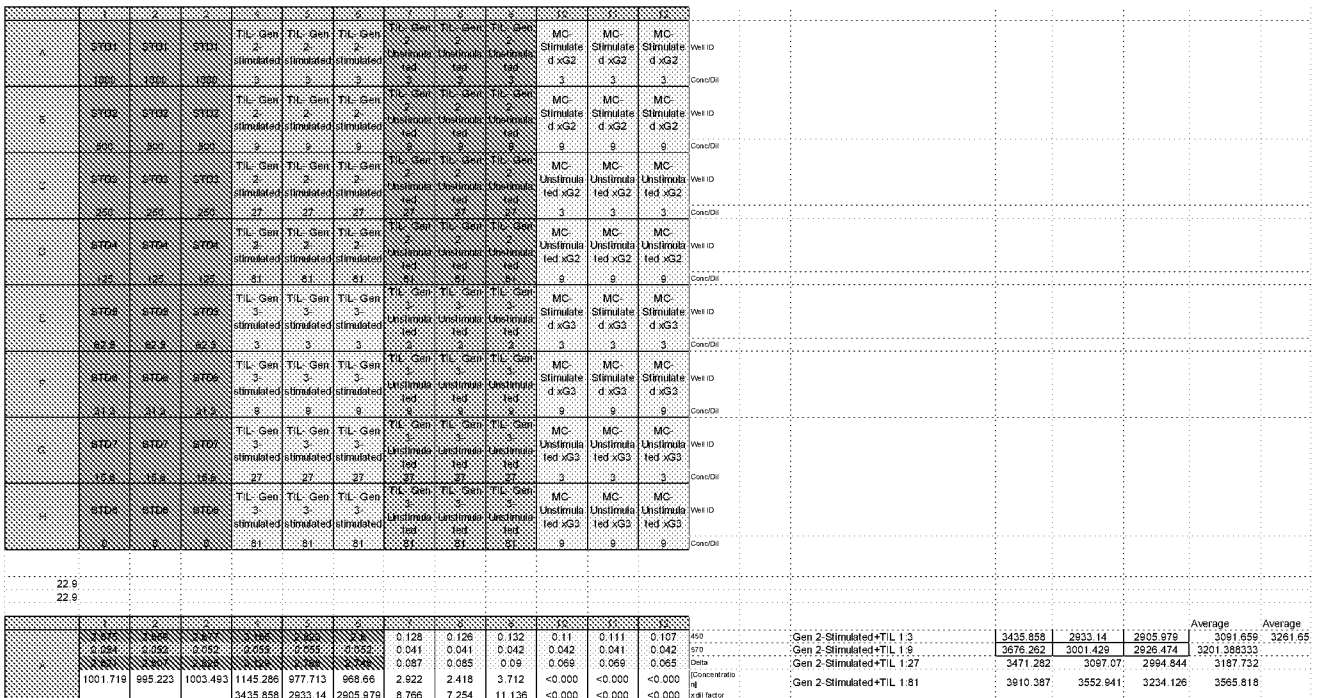


Figure 32

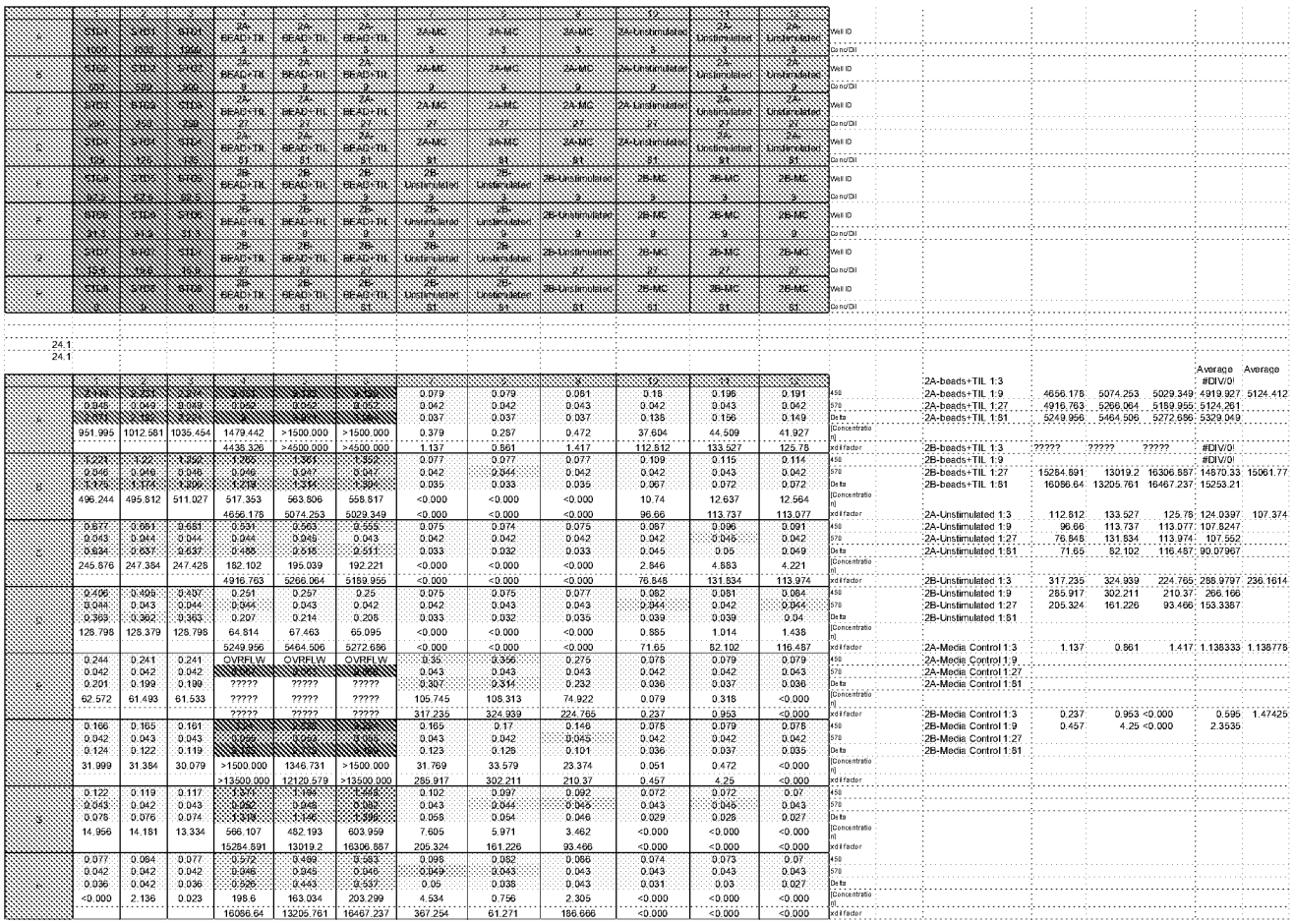


Figure 33

	1	2	3	4	5	6	7	8	9	10	11	12	
A	STD1 2000	STD1 2000	STD1 2000	Gen 3-D7 1ST RUN	Gen 3-D7 1ST RUN	Gen 3-D7 1ST RUN	Gen 3-D7 2ND RUN	Gen 3-D7 2ND RUN	Gen 3-D7 2ND RUN				Well ID Conc/Dil
B	STD2 1000	STD2 1000	STD2 1000	Gen 3-D11 1ST RUN	Gen 3-D11 1ST RUN	Gen 3-D11 1ST RUN	Gen 3-D11 2ND RUN	Gen 3-D11 2ND RUN	Gen 3-D11 2ND RUN				Well ID Conc/Dil
C	STD3 500	STD3 500	STD3 500	Gen 3-D16 1ST RUN	Gen 3-D16 1ST RUN	Gen 3-D16 1ST RUN	Gen 3-D16 2ND RUN	Gen 3-D16 2ND RUN	Gen 3-D16 2ND RUN				Well ID Conc/Dil
D	STD4 250	STD4 250	STD4 250	Gen 2-D11 1ST RUN	Gen 2-D11 1ST RUN	Gen 2-D11 1ST RUN	Gen 2-D11 2ND RUN	Gen 2-D11 2ND RUN	Gen 2-D11 2ND RUN				Well ID Conc/Dil
E	STD5 125	STD5 125	STD5 125	Gen 2-D16 1ST RUN	Gen 2-D16 1ST RUN	Gen 2-D16 1ST RUN	Gen 2-D16 2ND RUN	Gen 2-D16 2ND RUN	Gen 2-D16 2ND RUN				Well ID Conc/Dil
F	STD6 62.5	STD6 62.5	STD6 62.5	Gen 2-D22 1ST RUN	Gen 2-D22 1ST RUN	Gen 2-D22 1ST RUN	Gen 2-D22 2ND RUN	Gen 2-D22 2ND RUN	Gen 2-D22 2ND RUN				Well ID Conc/Dil
G	STD7 31.3	STD7 31.3	STD7 31.3	Empty	Empty	Empty	Empty	Empty	Empty				Well ID Conc/Dil
H	STD8 0	STD8 0	STD8 0	Empty	Empty	Empty	Empty	Empty	Empty				Well ID Conc/Dil

22
22

	1	2	3	4	5	6	7	8	9	10	11	12	
A	[illegible]	[illegible]	[illegible]	0.218	0.22	0.215	0.187	0.187	0.184				450
	[illegible]	[illegible]	[illegible]	0.043	0.042	0.043	0.044	0.043	0.051				570
	[illegible]	[illegible]	[illegible]	0.175	0.177	0.172	0.143	0.145	0.133				Delta
	1970.541	2046.905	1987.758	124.53	126.082	121.662	99.302	100.904	92.083				[Concentration]
B	1.095	1.281	1.276	0.415	0.412	0.435	0.394	0.392	0.356				450
	0.047	0.046	0.047	0.043	0.043	0.043	0.043	0.044	0.044				570
	1.048	1.234	1.229	0.372	0.369	0.393	0.351	0.348	0.312				Delta
	877.734	1051.728	1047.027	282.4	280.328	299.854	264.954	262.893	233.453				[Concentration]
C	0.683	0.682	0.68	0.379	0.372	0.376	0.356	0.338	0.33				450
	0.045	0.044	0.044	0.043	0.043	0.043	0.043	0.043	0.043				570
	0.638	0.639	0.637	0.336	0.33	0.333	0.313	0.295	0.287				Delta
	510.008	510.619	508.261	252.774	247.768	250.557	233.943	219.211	212.888				[Concentration]
D	0.37	0.38	0.382	0.162	0.156	0.177	0.155	0.15	0.149				450
	0.043	0.045	0.043	0.046	0.042	0.042	0.043	0.044	0.043				570
	0.328	0.335	0.338	0.116	0.114	0.135	0.112	0.106	0.106				Delta
	246.046	252.035	254.91	79.199	77.702	93.523	76.431	71.588	71.737				[Concentration]
E	0.216	0.219	0.22	0.235	0.227	0.228	0.253	0.231	0.222				450
	0.043	0.043	0.045	0.044	0.043	0.043	0.048	0.045	0.045				570
	0.173	0.176	0.175	0.191	0.185	0.185	0.205	0.186	0.177				Delta
	122.514	124.995	123.909	136.754	131.526	131.682	147.807	132.695	125.539				[Concentration]
F	0.137	0.132	0.133	0.21	0.227	0.216	0.238	0.233	0.253				450
	0.047	0.043	0.044	0.043	0.044	0.044	0.043	0.043	0.045				570
	0.089	0.089	0.089	0.166	0.183	0.172	0.195	0.19	0.207				Delta
	59.482	58.969	59.116	117.564	130.125	121.895	139.491	135.738	149.459				[Concentration]
G	0.088	0.088	0.089	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]				450
	0.042	0.043	0.043	[illegible]	0.05	[illegible]	[illegible]	[illegible]	[illegible]				570
	0.046	0.046	0.046	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]				Delta
	28.375	28.237	28.514	2014.9	2081.651	1970.439	1973.799	1920.845	1986.127				[Concentration]
H	0.05	0.048	0.048	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]				450
	0.043	0.042	0.041	[illegible]	0.05	[illegible]	[illegible]	[illegible]	[illegible]				570
	0.007	0.006	0.007	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]				Delta
	3.1	2.399	2.573	2023.073	2016.943	1960.16	1986.84	2002.036	1988.165				[Concentration]

Dilution Factor=100

	Reading A	Reading B	Reading C	Reading A*100	Reading B*100	Reading C*100	Average
Gen 3 Rep initiation 1st run	124.530	126.082	121.662	12453.0	12608.2	12166.2	12409.1
Gen 3 Scale up 1st run	282.400	280.328	299.854	28240.0	28032.8	29985.4	28752.7
Gen 3 Harvest 1st run	252.774	247.768	250.557	25277.4	24776.8	25055.7	25036.6
Gen 2 Rep initiation 1st run	79.199	77.702	93.523	7919.9	7770.2	9352.3	8347.5
Gen 2 Scale up 1st run	136.754	131.526	131.682	13675.4	13152.6	13168.2	13332.1
Gen 2 Harvest 1st run	117.564	130.125	121.895	11756.4	13012.5	12189.5	12319.5
Gen 3 Rep initiation 2nd run	99.302	100.904	92.083	9930.2	10090.4	9208.3	9743.0
Gen 3 Scale up 2nd run	264.954	262.893	233.453	26495.4	26289.3	23345.3	25376.7
Gen 3 Harvest 2nd run	233.943	219.211	212.888	23394.3	21921.1	21288.8	22201.4
Gen 2 Rep initiation 2nd run	76.431	71.588	71.737	7643.1	7158.8	7173.7	7325.2
Gen 2 Scale up 2nd run	147.807	132.695	125.539	14780.7	13269.5	12553.9	13534.7
Gen 2 Harvest 2nd run	139.491	135.738	149.459	13949.1	13573.8	14945.9	14156.3

Figure 34

Sample number #	Description	Glucose (g/L)	Lactate (g/L)	Ammonia (mmol/L)	Glutamine (mmol/L)	Glutamax (mmol/L)	Glutamax-Glutamine (mmol/L)
1	Gen 2-Rep initiation First Round #L4054	1.78	0.14	2.16	1.56	1.71	0.16
2	Gen 2-Scale Up First Round #L4054	1.36	0.89	1.40	1.76	1.90	0.15
3	Gen 2-Harvest First Round #L4054	0.83	1.68	2.46	1.69	1.85	0.17
4	Gen 3-REP initiation First Round #L4054	1.68	0.29	1.76	2.11	2.29	0.18
5	Gen 3-Scale Up First Round #L4054	1.28	0.94	1.64	1.70	1.87	0.16
6	Gen 3-Harvest First Round #L4054	1.07	1.53	2.23	1.39	1.54	0.14
7	Gen 2-Rep initiation Second Round #L4055	1.91	0.08	2.29	1.60	1.74	0.14
8	Gen 2-Scale up Second Round #L4055	1.61	0.77	1.40	1.78	1.93	0.15
9	Gen 2-Harvest Second Round #L4055	0.85	1.86	2.46	1.39	1.55	0.16
10	Gen 3-REP initiation Second Round #L4055	1.77	0.18	1.74	2.06	2.23	0.17
11	Gen 3-Scale up Second Round #L4055	1.60	0.72	1.54	1.68	1.84	0.15
12	Gen 3-Harvest Second Round #L4055	1.44	1.32	2.07	1.79	1.95	0.16

Figure 35

	$FIL	S/L/1301 | Geometr	S/L/Pt | Geometric Mean (FL03-A)
A1 gen2L4054 ne	A1 gen2L4054 neg.fcs	34.5	3.24
A2 gen2L4054 ne	A2 gen2L4054 neg.fcs	34	3.19
A3 gen2L4054 po	A3 gen2L4054 pos.fcs	1834	70.6
A4 gen2L4054 po	A4 gen2L4054 pos.fcs	1872	70.3
A5 gen2L4055 ne	A5 gen2L4055 neg.fcs	31.9	4.23
A6 gen2L4055 ne	A6 gen2L4055 neg.fcs	32.4	4.87
A7 gen2L4055 po	A7 gen2L4055 pos.fcs	1941	88.7
A8 gen2L4055 po	A8 gen2L4055 pos.fcs	1933	91.1
B1 gen3L4054 ne	B1 gen3L4054 neg.fcs	31.2	3.45
B2 gen3L4054 ne	B2 gen3L4054 neg.fcs	31.9	3.39
B3 gen3L4054 po	B3 gen3L4054 pos.fcs	2016	61.3
B4 gen3L4054 po	B4 gen3L4054 pos.fcs	2014	76.2
B5 gen3L4055 ne	B5 gen3L4055 neg.fcs	33.7	6.76
B6 gen3L4055 ne	B6 gen3L4055 neg.fcs	35.4	6.7
B7 gen3L4055 po	B7 gen3L4055 pos.fcs	2158	99.4
B8 gen3L4055 po	B8 gen3L4055 pos.fcs	1938	99.5
Mean	-	998	43.3
SD	-	999	41.3

Figure 36

Study	Sample	Sample id	Species	Chain	Reads	CDR3	Unique CDR3	D50
20180511_Study1_FCA	Gen2-L4054	59990	TRB	h	1181732	1181732	8915	0
20180511_Study1_FCA	Gen3-L4054	59991	TRB	h	1145697	1145697	18130	0
20180511_Study1_FCA	Gen2-L4055	59987	TRB	h	1166465	1166465	12996	0.1
20180511_Study1_FCA	Gen3-L4055	59982	TRB	h	1059985	1059985	27246	0.9

Figure 37

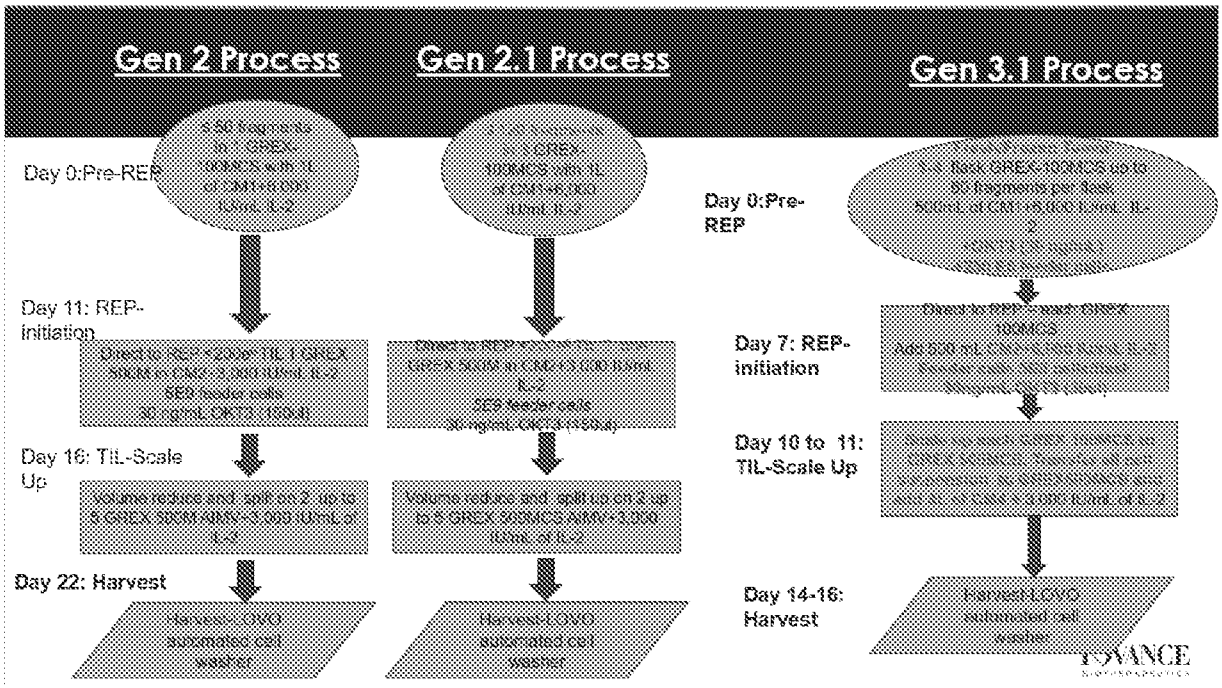

Gen 2 Process
Gen 2.1 Process
Gen 3.1 Process
Day 0:Pre-REP
Day 11: REP-Initiation
Day 16: TIL-Scale Up
Day 22: Harvest
Day 0:Pre-REP
Day 7: REP-initiation
Day 10 to 11: TIL-Scale Up
Day 14-16: Harvest
Harvest LOVO automated cell washer
Harvest LOVO automated cell washer
Harvest LOVO automated cell washer
IOVANCE

Figure 38

STEP	Gen 2	Gen 2.1	Gen 3.0 Optimized
Pre REP- day 0	≤ 50 fragments/ 1 G-Rex 100MCS - **11 days**	≤ 150 fragments/ 3 G-Rex Pre-formulated CM1 warmed media 100MCS - **11 days**	***Fresh or Frozen Tumor*** Whole tumor with ≤ 50 fragments up to 80 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 (3 wash cycle)	**Harvest day 14 or 16** LOVO- automated cell washer (3 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 IL-2-CS10 in LN_2 multiple aliquots
Process time	22 days	22 days	18 days

Figure 39

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 40

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 doubling 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 41

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 42

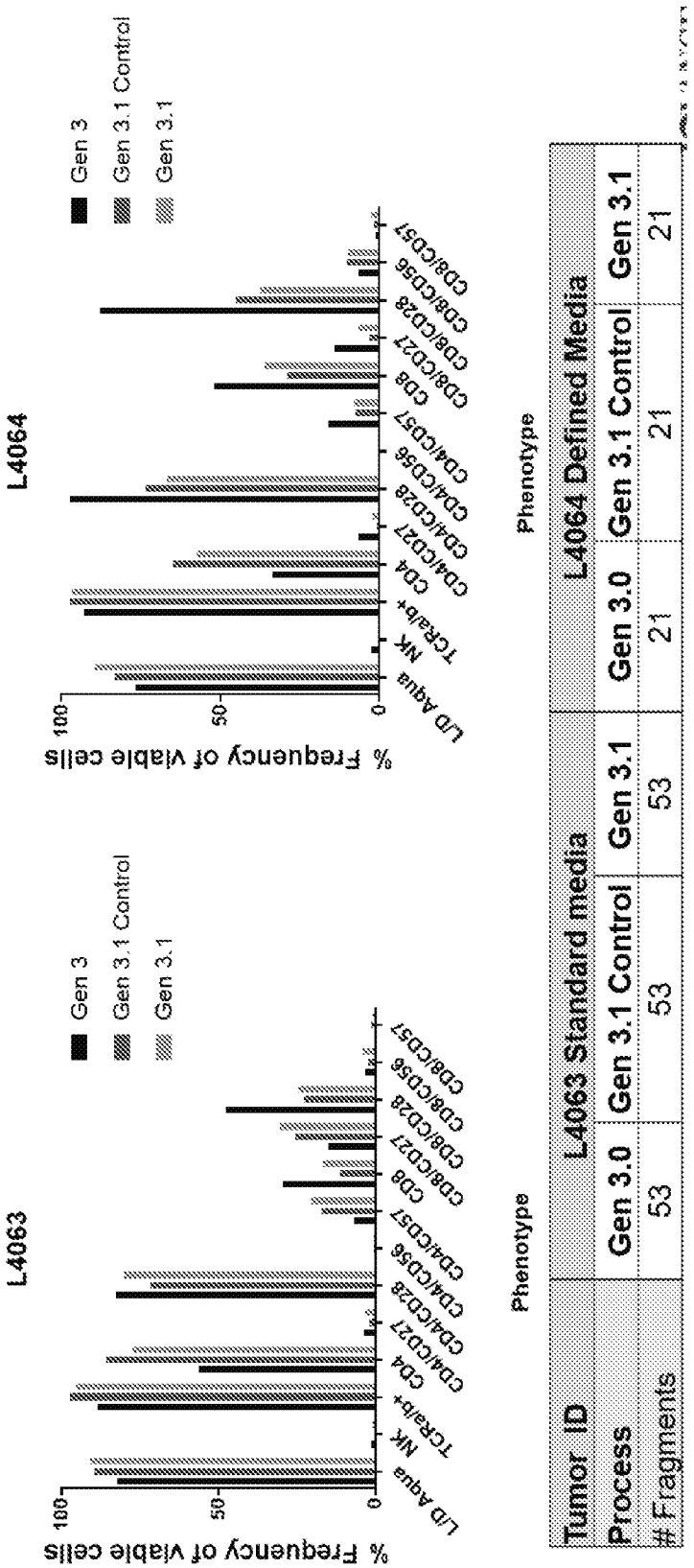
Process	Gen 2	Gen 3
L4054	Standard Media	Standard Media
L4055	Standard Media	Standard Media
M10851	Standard Media	Standard Media

Process	Gen 3	Gen 3.1 control	Gen 3.1
L4063	Standard Media	Standard Media	Standard Media
L4064	Defined Media	Defined Media	Defined Media

Standard Media:
Pre REP: CM1
REP initiation : CM2
Split or Scale up : CM4

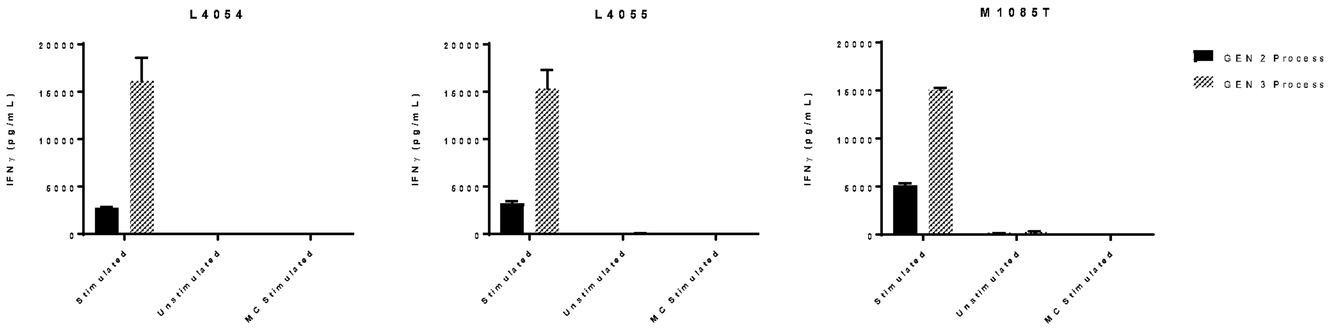
Defined Media:
CTS Optimizer (Serum Free Media) in each day of the process

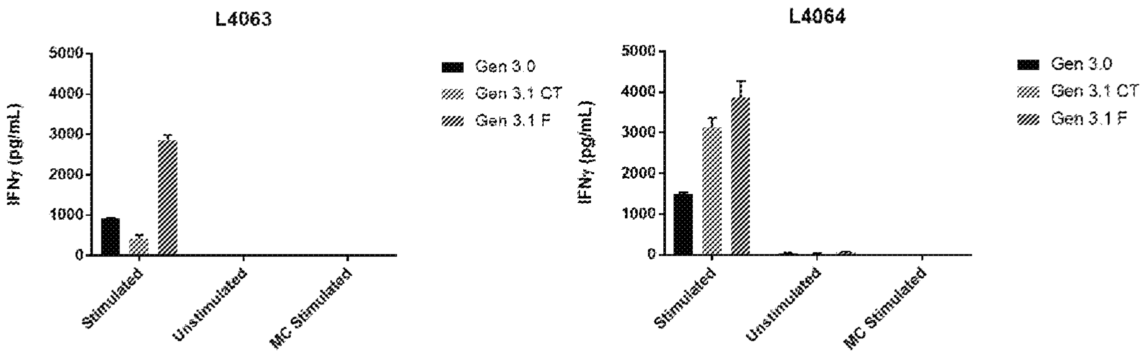
Figure 43



Tumor ID	L4063 Standard media			L4064 Defined Media		
Process	Gen 3.0	Gen 3.1 Control	Gen 3.1	Gen 3.0	Gen 3.1 Control	Gen 3.1
# Fragments	53	53	53	21	21	21

Figure 44





L4063- Standard Media:

L4064- Defined Media

Process Day	Conditions	Gen 3.0	Gen 3.1 CT (control)	Gen 3.1 F
Day 0- pre-REP initiation	Media CM1	500 mL	500 mL	500 mL
	IL-2 (6000 IU/mL)	+	+	+
	OKT-3 (30ng/mL)	-	+	+
	Feeders (250 E+06)	-	-	+

Figure 45

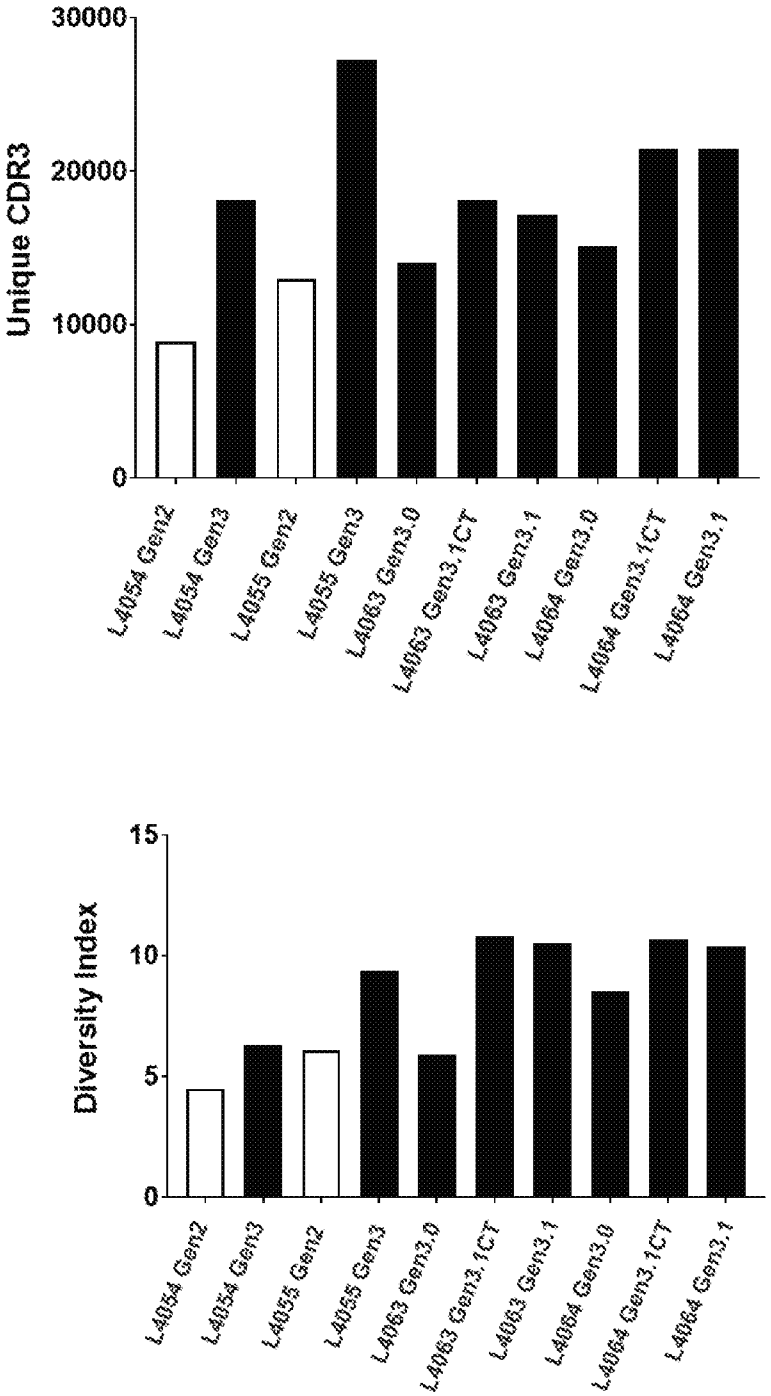

Unique CDR3
30000
20000
10000
0
L4054 Gen2
L4054 Gen3
L4055 Gen2
L4055 Gen3
L4063 Gen3.0
L4063 Gen3.1CT
L4063 Gen3.1
L4064 Gen3.0
L4064 Gen3.1CT
L4064 Gen3.1
Diversity Index
15
10
5
0
L4054 Gen2
L4054 Gen3
L4055 Gen2
L4055 Gen3
L4063 Gen3.0
L4063 Gen3.1CT
L4063 Gen3.1
L4064 Gen3.0
L4064 Gen3.1CT
L4064 Gen3.1

Figure 46

Conditions on Day 0	Gen 3.0	Gen 3.1 (control)	Gen 3.1
Media	500 mL	500 mL	500 mL
IL-2 (6E+06)	+	+	+
OKT-3 (30ng/mL)	-	+	+
Feeders (250 E+06)	-	-	+

	All uCDR3's			Top 80% uCDR3's		
Number of uCDR3 (% Overlap)	Gen 3.0	Gen 3.1 Control	Gen 3.1	Gen 3.1	Gen 3.1 control	Gen 3.1
Gen 3.0	14005	3240 (23)	2959 (21)	1105	1000 (90)	975 (88)
Gen 3.1 control	-	18149	-	-	2438	-
Gen 3.1	-	-	17181	-	-	2332

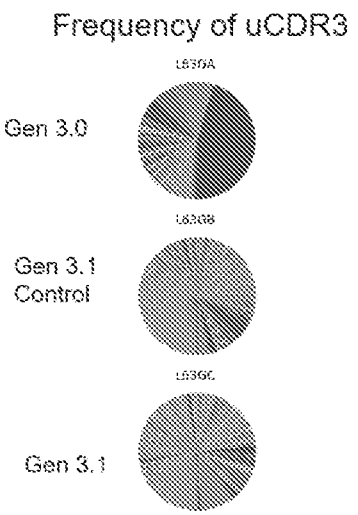


Figure 47

Conditions on Day 0	Gen 3.0	Gen 3.1 (control)	Gen 3.1
Media	500 mL	500 mL	500 mL
IL-2 (6E+06)	+	+	+
OKT-3 (30ng/mL)	-	+	+
Feeders (250 E+06)	-	-	+

Number of uCDR3 (% Overlap)	All uCDR3's			Top 80% uCDR3's		
	Gen 3.0	Gen 3.1 Control	Gen 3.1	Gen 3.0	Gen 3.1 Control	Gen 3.1
Gen 3.0	15107	5486 (36)	5541 (37)	2479	2186 (88)	2163 (87)
Gen 3.1 Control	-	21460	-	-	3247	-
Gen 3.1	-	-	21503	-	-	3264

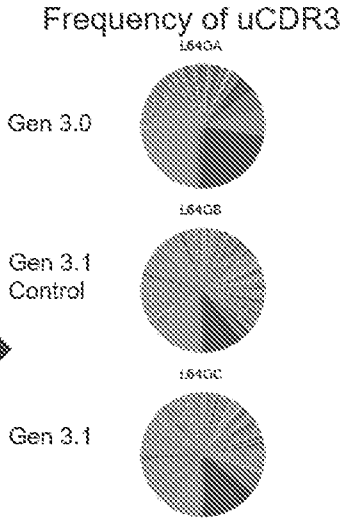


Figure 48

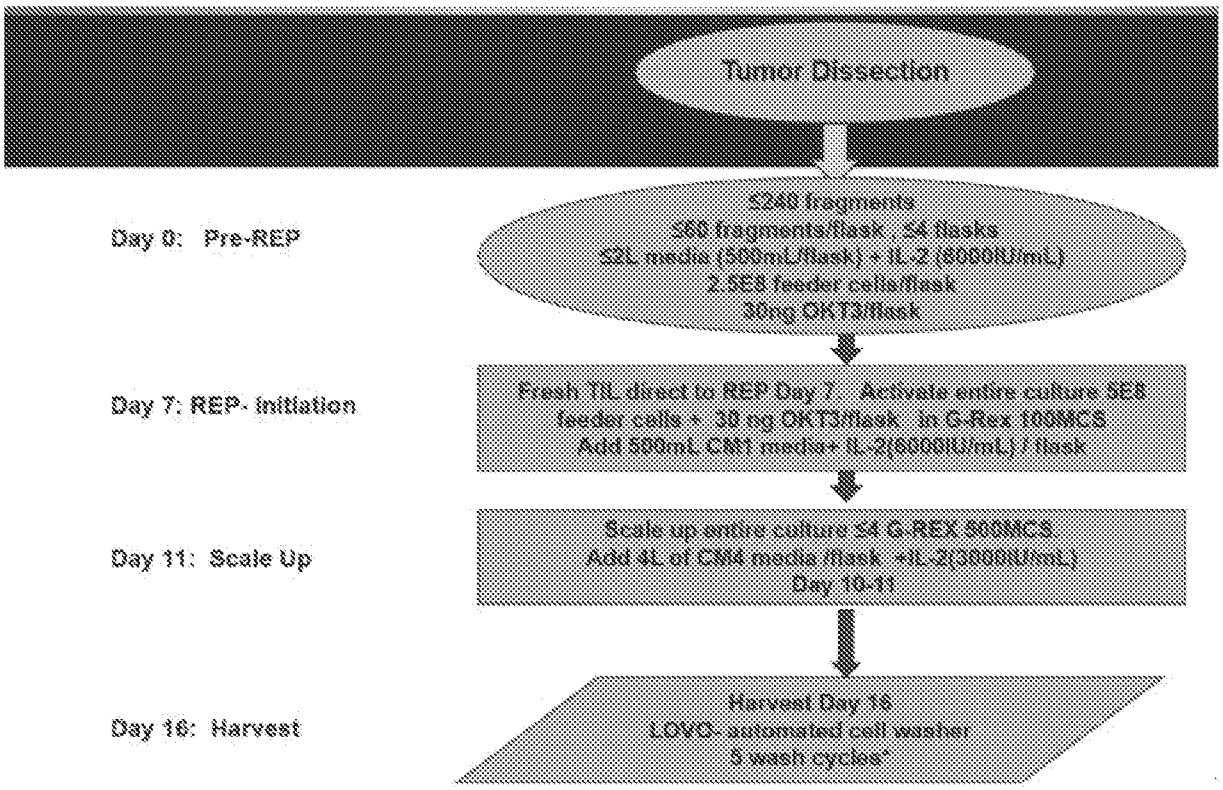

Tumor Dissection
Day 0: Pre-REP
≤240 fragments
≤60 fragments/flask , ≤4 flasks
≤2L media (500mL/flask) + IL-2 (6000IU/mL)
2.5E8 feeder cells/flask
30ng OKT3/flask
Day 7: REP- Initiation
Fresh TIL direct to REP Day 7 Activate entire culture 5E8
feeder cells + 30 ng OKT3/flask in G-Rex 100MCS
Add 500mL CM1 media+ IL-2(6000IU/mL) / flask
Day 11: Scale Up
Scale up entire culture ≤4 G-REX 500MCS
Add 4L of CM4 media /flask +IL-2(3000IU/mL)
Day 10-11
Day 16: Harvest
Harvest Day 16
LOVO- automated cell washer
5 wash cycles*

Figure 49

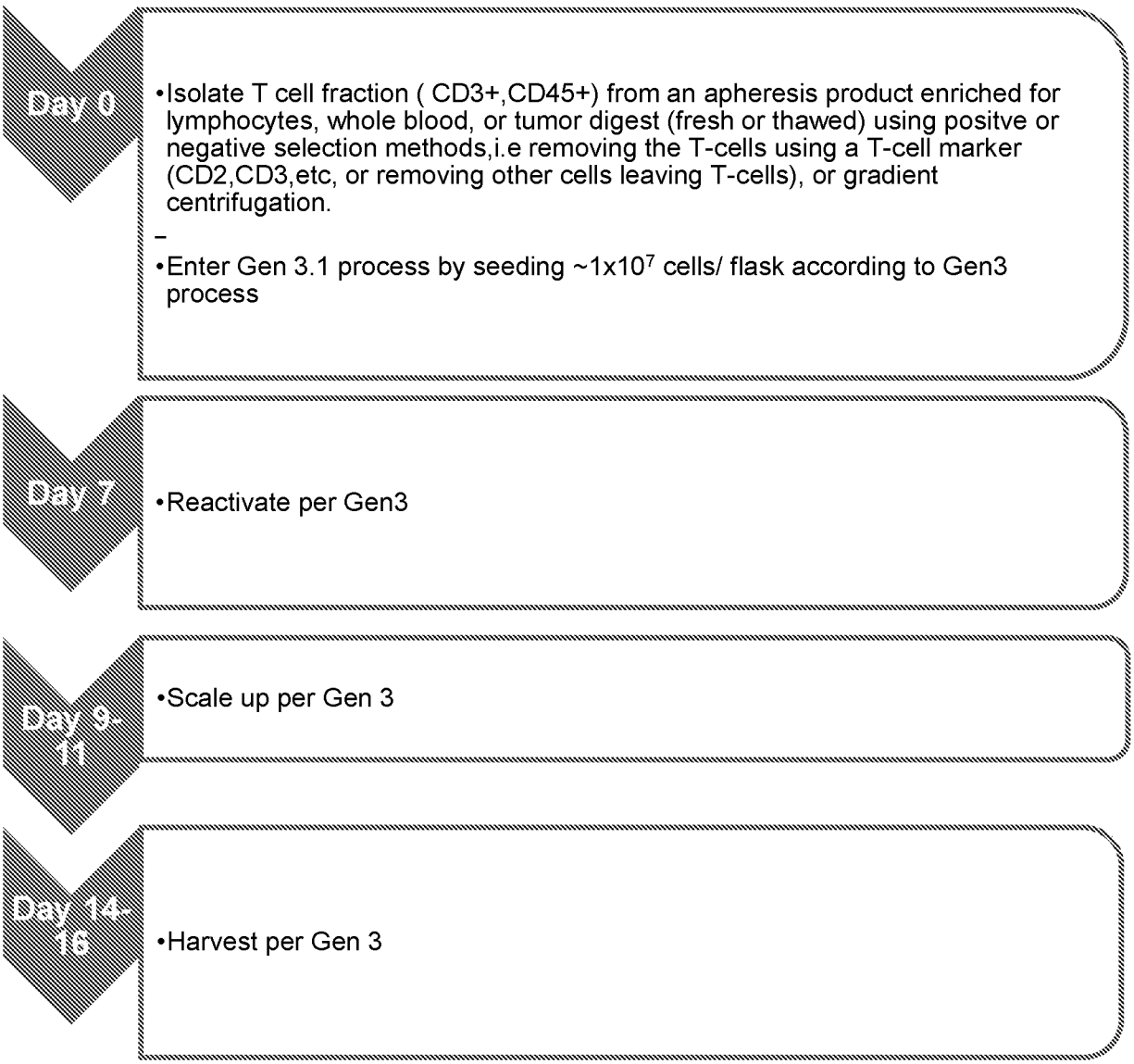

Day 0
•Isolate T cell fraction (CD3+,CD45+) from an apheresis product enriched for lymphocytes, whole blood, or tumor digest (fresh or thawed) using positve or negative selection methods,i.e removing the T-cells using a T-cell marker (CD2,CD3,etc, or removing other cells leaving T-cells), or gradient 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 50

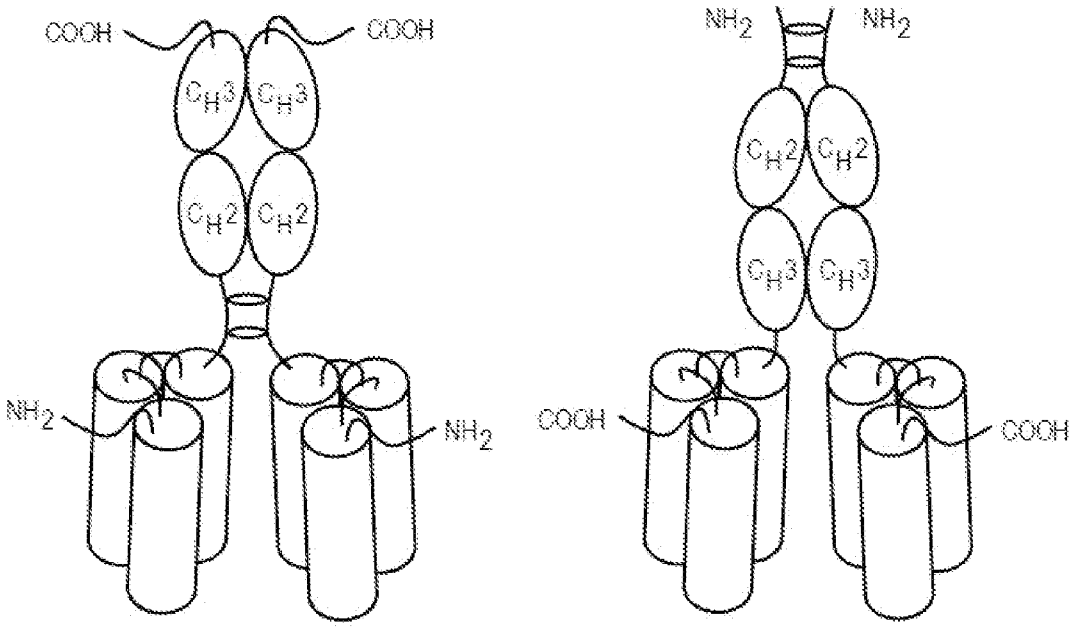

COOH
COOH
C_H3
C_H3
C_H2
C_H2
NH_2
NH_2
Structure I-A
NH_2
NH_2
C_H2
C_H2
C_H3
C_H3
COOH
COOH
Structure I-B

Figure 51

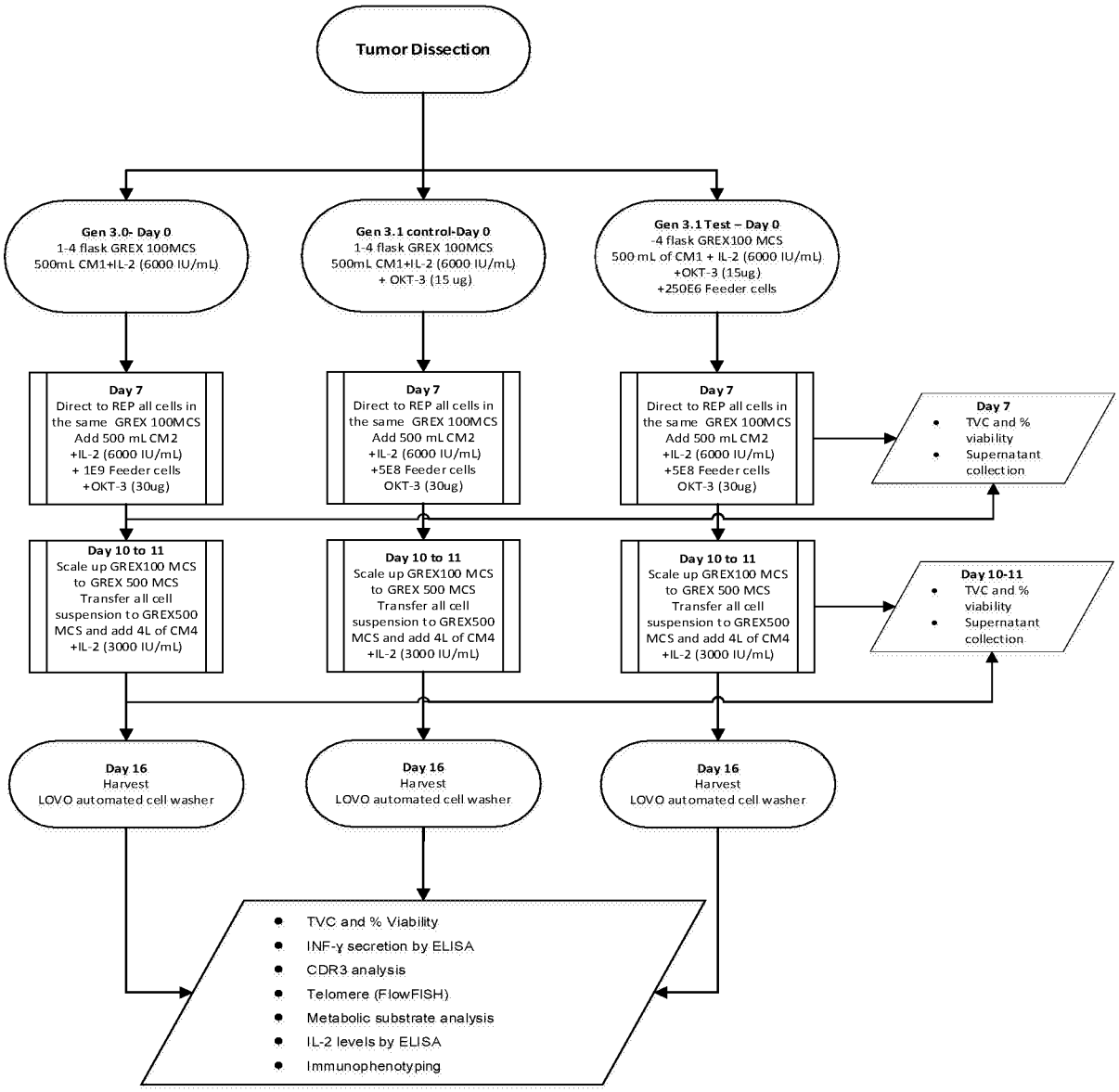
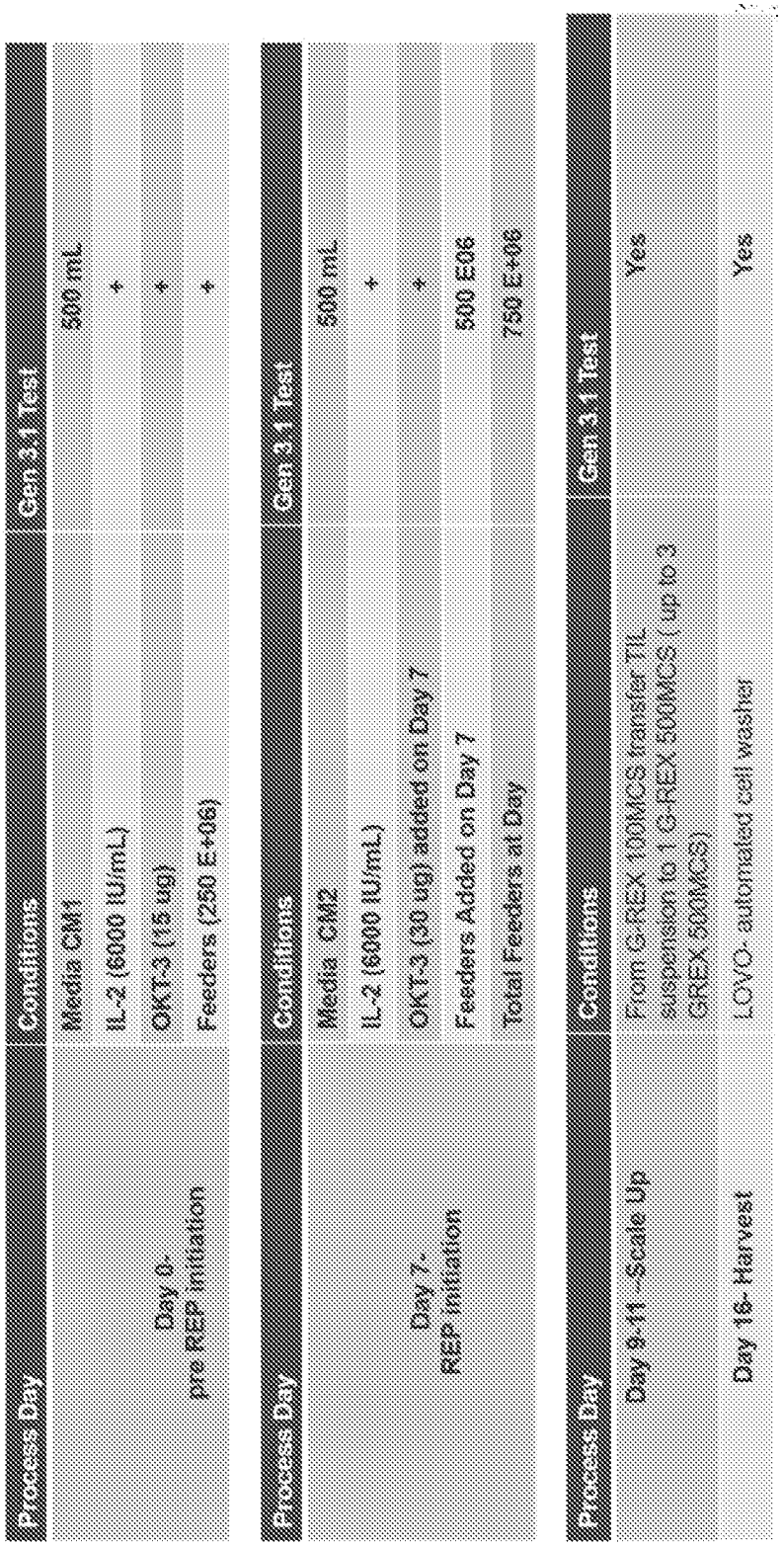

Tumor Dissection
Gen 3.0- Day 0
1-4 flask GREX 100MCS
500mL CM1+IL-2 (6000 IU/mL)
Gen 3.1 control-Day 0
1-4 flask GREX 100MCS
500mL CM1+IL-2 (6000 IU/mL)
+ OKT-3 (15 ug)
Gen 3.1 Test – Day 0
-4 flask GREX100 MCS
500 mL of CM1 + IL-2 (6000 IU/mL)
+OKT-3 (15ug)
+250E6 Feeder cells
Day 7
Direct to REP all 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 52



Process Day	Conditions	Gen 3.1 Test
Day 0- pre REP initiation	Media CM1	500 mL
	IL-2 (6000 IU/mL)	+
	OKT-3 (15 ug)	+
	Feeders (250 E+06)	+

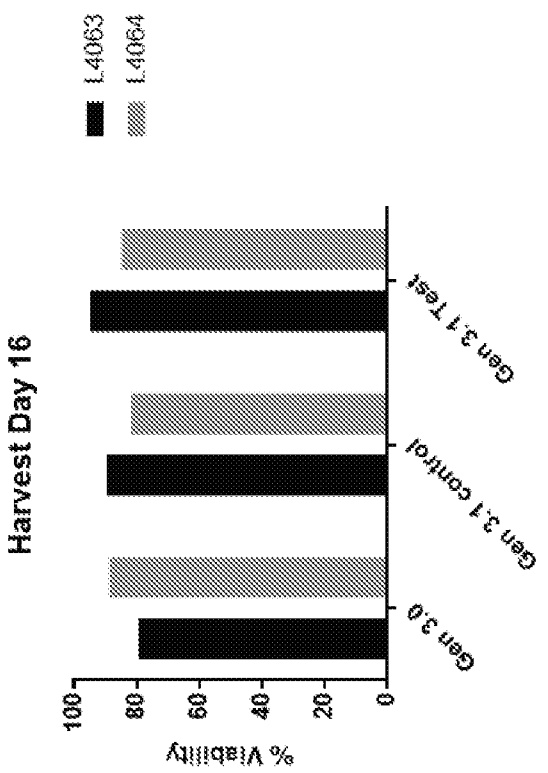
Process Day	Conditions	Gen 3.1 Test
Day 7- REP initiation	Media CM2	500 mL
	IL-2 (6000 IU/mL)	+
	OKT-3 (30 ug) added on Day 7	+
	Feeders Added on Day 7	500 E06
	Total Feeders at Day	750 E+06

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 53

Tumor ID	L4063 in Standard Media			L4064 in CTS Optimizer Media		
Process	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Number of fragments	53	53	53	21	21	21
Average TVC per fragment	1.37E+08	3.19E+08	3.53E+08	5.90E+08	7.57E+08	9.29E+08
% viability at Harvest	79.53%	89.43%	94.80%	88.80%	81.90%	84.90%
TVC Harvest	**7.26E+09**	**1.69E+10**	**1.87E+10 ***	**1.24E+10**	**1.59E+10**	**1.95E+10 ***

Figure 54


L4063
L4064
Harvest Day 16
% Viability
100
80
60
40
20
0
Gen 3.0
Gen 3.1 control
Gen 3.1 Test

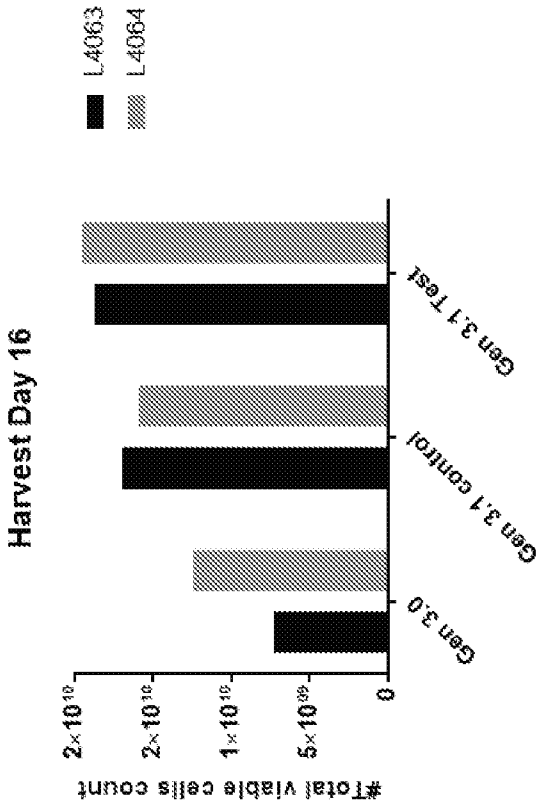
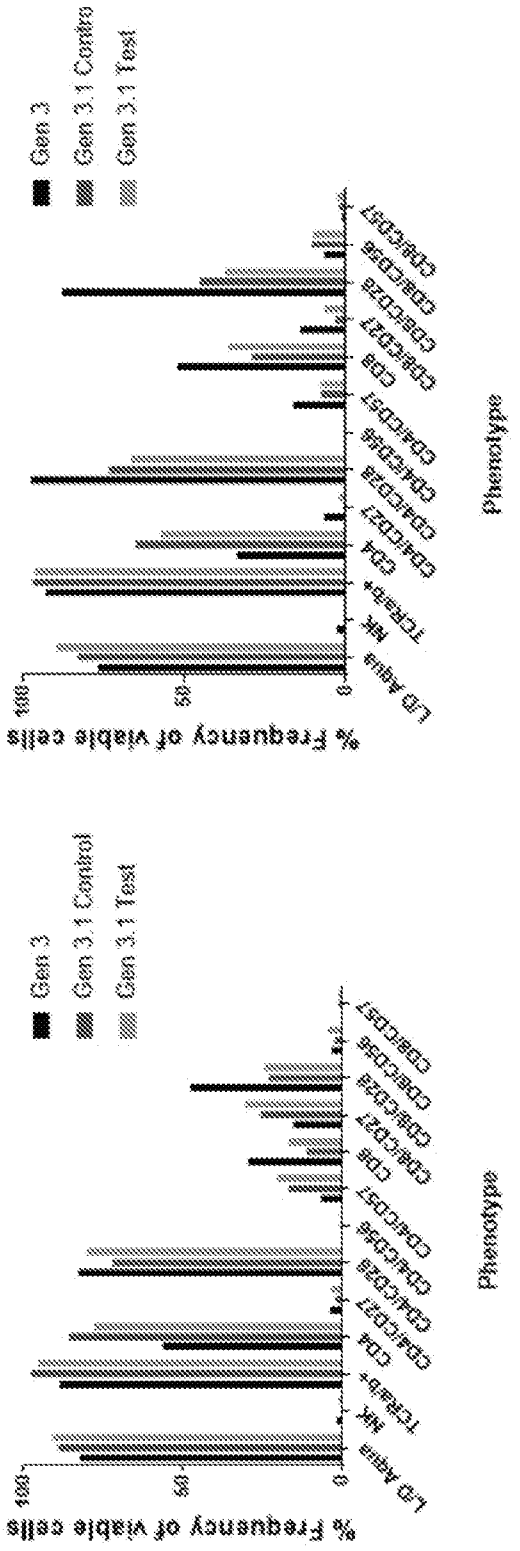

L4063
L4064
Harvest Day 16
#Total viable cells count
2×10^10
2×10^10
1×10^10
5×10^09
0
Gen 3.0
Gen 3.1 control
Gen 3.1 Test

Figure 55



Tumor ID	L4063 Standard media			L4064 Defined Media		
Process	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
# Fragments	53	53	53	21	21	21

Figure 56

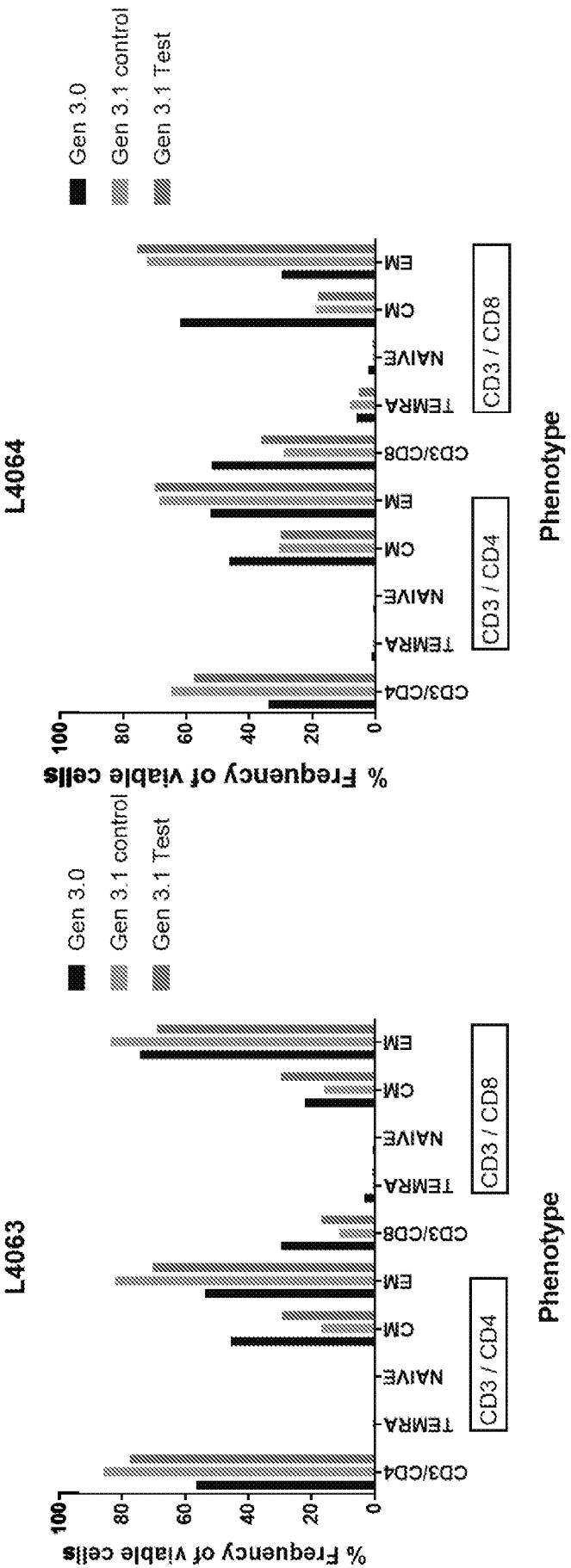

Gen 3.0
Gen 3.1 control
Gen 3.1 Test
L4064
% Frequency of viable cells
100
80
60
40
20
0
CD3/CD4
TEMRA
NAIVE
CM
EM
CD3/CD8
CD3 / CD4
CD3 / CD8
Phenotype
Gen 3.0
Gen 3.1 control
Gen 3.1 Test
L4063
% Frequency of viable cells
100
80
60
40
20
0
CD3/CD4
TEMRA
NAIVE
CM
EM
CD3/CD8
CD3 / CD4
CD3 / CD8
Phenotype

Figure 57

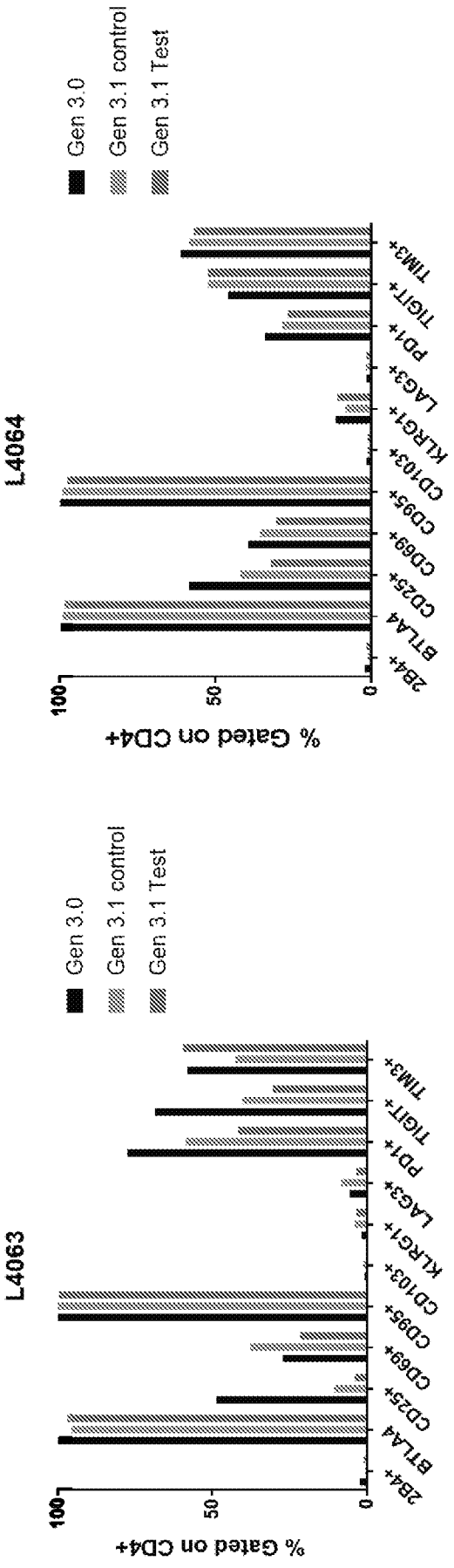

L4064
Gen 3.0
Gen 3.1 control
Gen 3.1 Test
% Gated on CD4+
100
50
0
2B4+
BTLA4
CD25+
CD69+
CD95+
CD103+
KLRG1+
LAG3+
PD1+
TIGIT+
TIM3+
L4063
Gen 3.0
Gen 3.1 control
Gen 3.1 Test
% Gated on CD4+
100
50
0
2B4+
BTLA4
CD25+
CD69+
CD95+
CD103+
KLRG1+
LAG3+
PD1+
TIGIT+
TIM3+

Figure 58

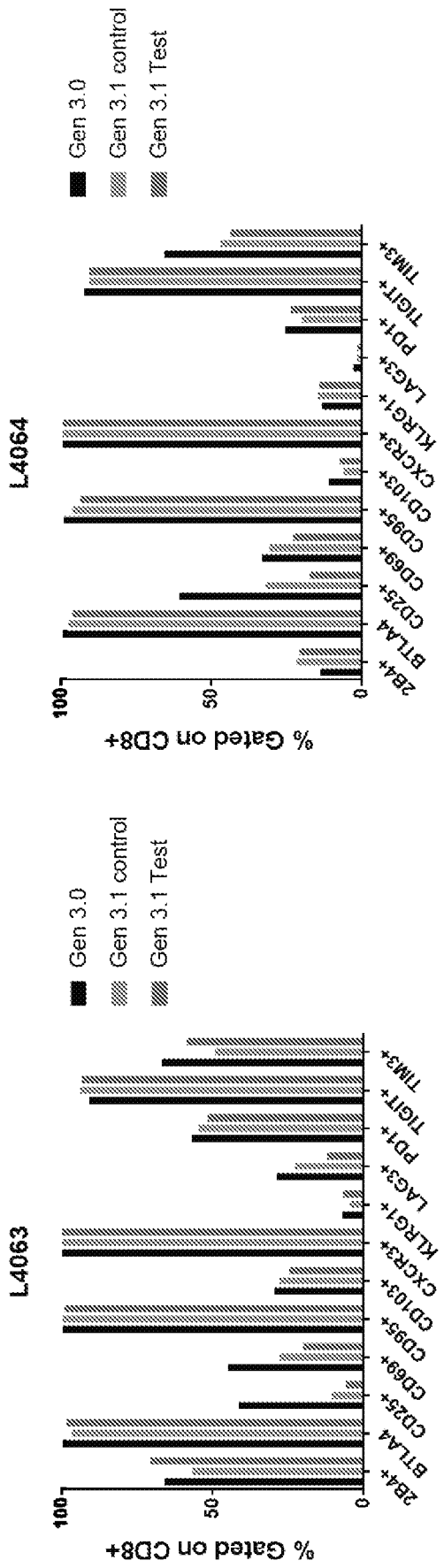
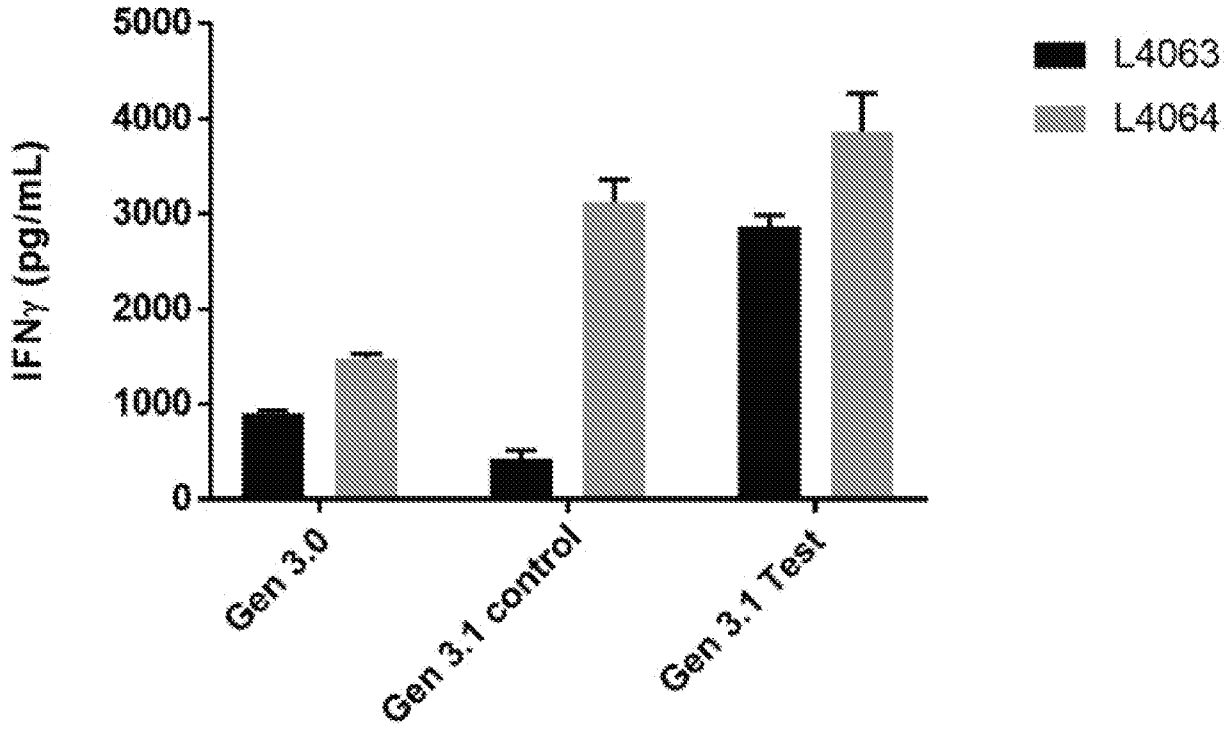

L4064
L4063
Gen 3.0
Gen 3.1 control
Gen 3.1 Test
% Gated on CD8+
100
50
0
2B4+
BTLA4
CD25+
CD69+
CD95+
CD103+
CXCR3+
KLRG1+
LAG3+
PD1+
TIGIT+
TIM3+

Figure 59



Process Day	Conditions	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Day 0- pre REP initiation	Media CM1	500 mL	500 mL	500 mL
	IL-2 (6000 IU/mL)	+	+	+
	OKT-3 (30ng/mL)	-	+	+
	Feeders (250 E+06)	-	-	+

Figure 60

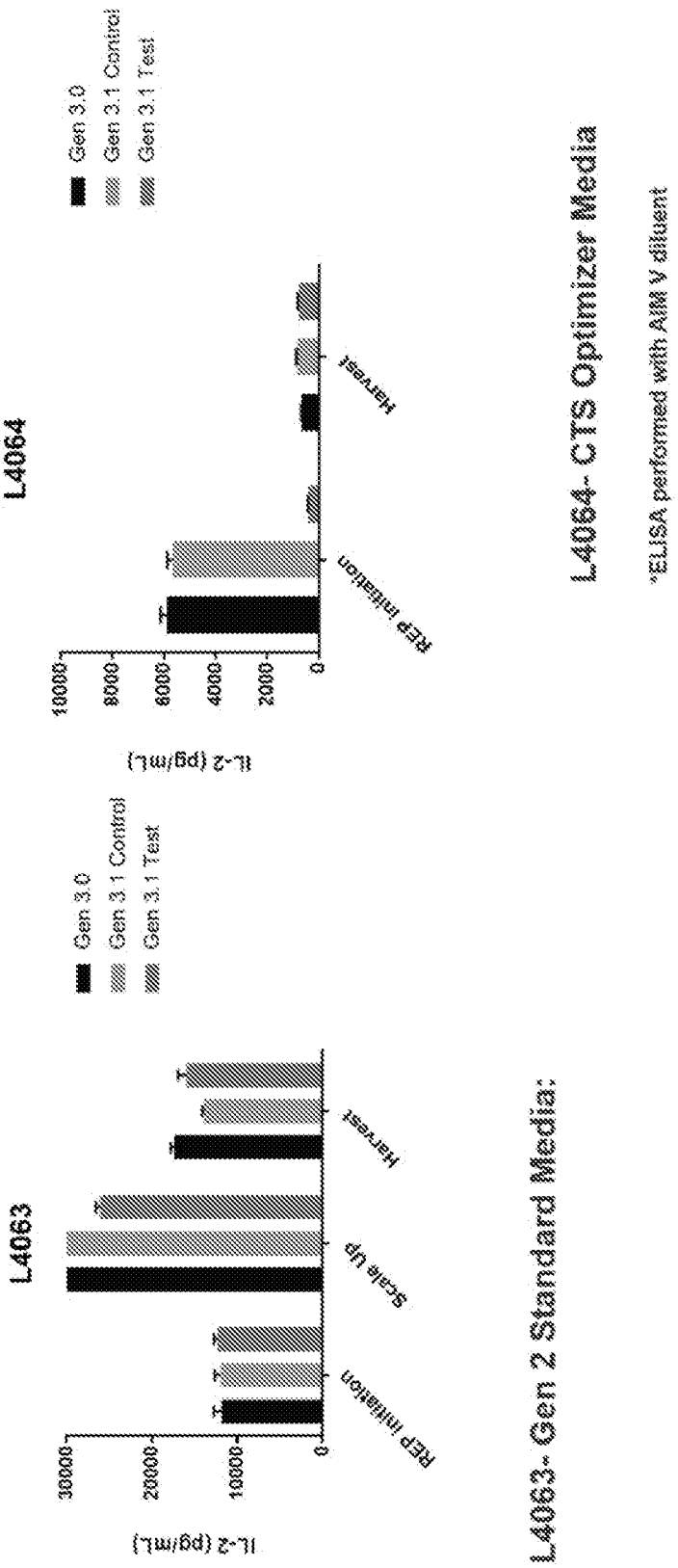

L4064
Gen 3.0
Gen 3.1 Control
Gen 3.1 Test
IL-2 (pg/mL)
10000
8000
6000
4000
2000
0
REP Initiation
Harvest
L4064- CTS Optimizer Media
*ELISA performed with AIM V diluent
L4063
Gen 3.0
Gen 3.1 Control
Gen 3.1 Test
IL-2 (pg/mL)
30000
20000
10000
0
REP Initiation
Scale Up
Harvest
L4063- Gen 2 Standard Media:

Figure 61

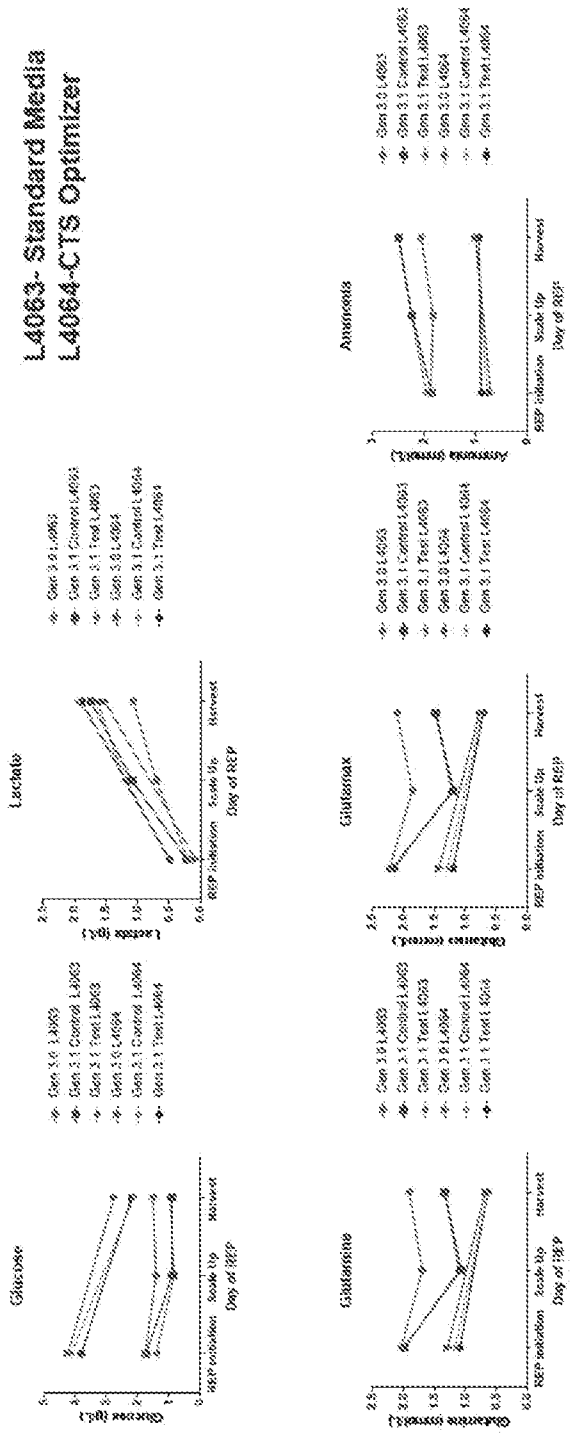

L4063- Standard Media
L4064-CTS Optimizer
Glucose
Lactate
Glutamine
Glutamax
Ammonia
REP initiation
Scale Up
Harvest
Day of REP

Figure 62

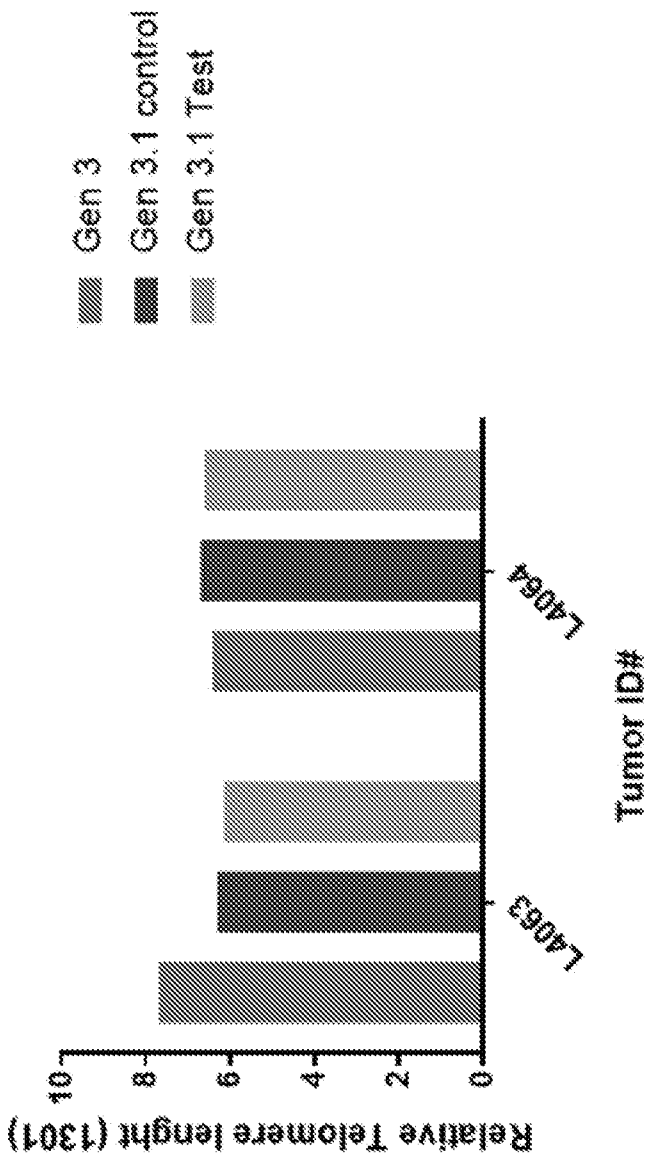

Gen 3
Gen 3.1 control
Gen 3.1 Test
Relative Telomere length (1301)
10
8
6
4
2
0
LA063
LA064
Tumor ID#

Figure 63

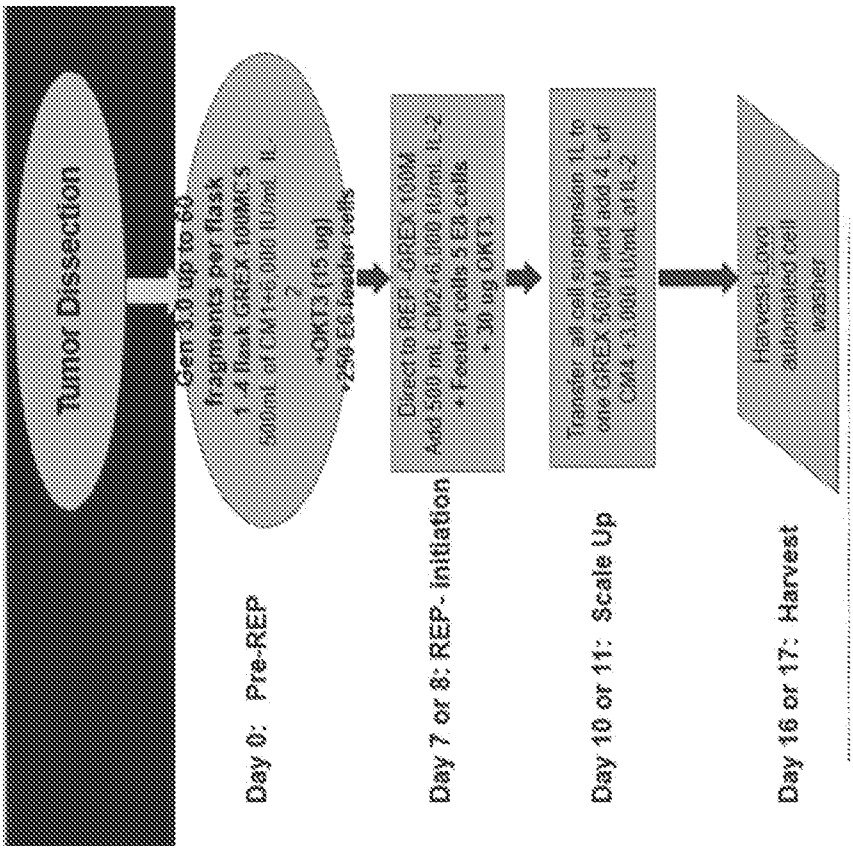

Tumor Dissection
Day 0: Pre-REP
Day 7 or 8: REP- initiation
Day 10 or 11: Scale Up
Day 16 or 17: Harvest

Figure 64

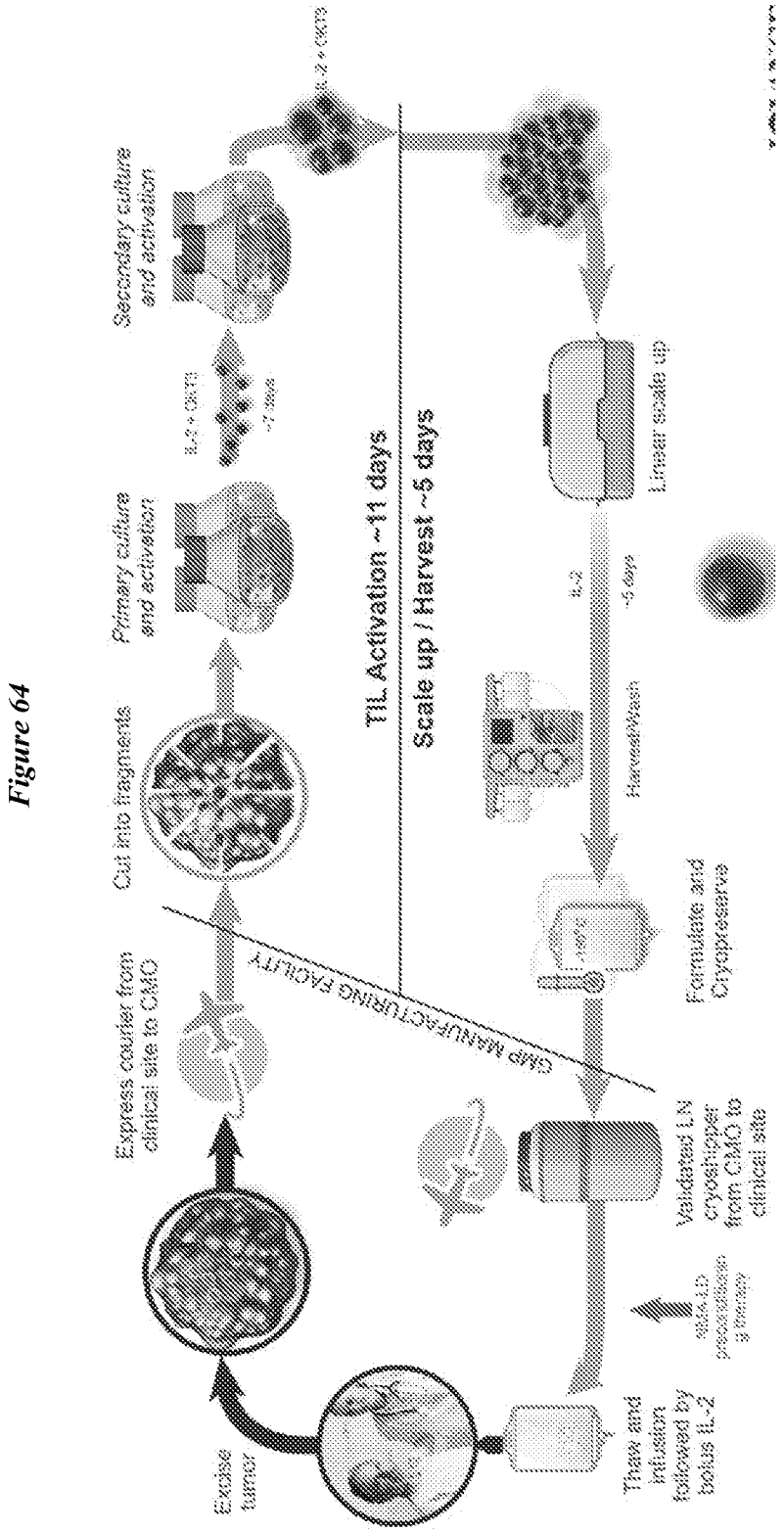

Excise tumor
Express courier from clinical site to CMO
Cut into fragments
Primary culture and activation
Secondary culture and activation
TIL Activation ~11 days
Scale up / Harvest ~5 days
GMP MANUFACTURING FACILITY
Linear scale up
Harvest/Wash
Formulate and Cryopreserve
Validated LN cryoshipper from CMO to clinical site
Thaw and infusion followed by bolus IL-2

Figure 65A

	Gen 2	Gen 3
Total Culture Time	22d	16-17d
Pre-REP		
Fragments/flask	≤60 fragments in 1 flask	≤60 fragments/flask in up to 4 flasks
Media volume	1L - Single addition	1L – 2 x 500mL additions
Target preREP cell numbers	≤200e6 TIL	All cells carried through continuous process
Screening	No screen	No screen
Selection of flasks	No selection	Bac-T sterility, visual inspection for contaminants
REP / Scale up		
Feeders		Reduced by ≥40%
Media	Contains HSAB	Defined
Scale up	Pooled culture Volume reduce to 500mL on Day 5 split up to 5 flasks (2500 cm2)	Flasks scaled linearly and treated as subcomponents
OKT3	150ug	≤180ug at max scale
IL2	High dose	High dose
Number of flasks	1-5	1-4
Harvest-Volume reduction	Closed 10:1	Closed 10:1
Concentrate/Wash	LOVO 100:1	LOVO 1000:1
Formulation	1:1 CS10 (5% DMSO)	1:1 CS10 (5% DMSO)
Shipment	Vapor phase LN	Vapor phase LN
Infusion	Thawed IV gravity	Thawed IV gravity

Figure 65B

	Gen 2	Gen 3
Total Culture Time	22d	16d
Fragment Culture		
Fragments/flask	≤60 fragments in 1 flask	≤60 fragments/flask in up to 4 flasks
Media volume	1L - Single addition	1L – 2 x 500mL additions
Target preREP cell numbers	≤230e6 TIL	All cells carried through continuous process
Screening	No screen	No screen
Selection of flasks	No selection	Bac-T sterility, visual inspection for contaminants
REP / Scale up		
Feeders		Reduced by ≥40%
Media	Contains HSAB	Defined
Scale up	Pooled culture Volume reduce to 500mL on Day 5 split up to 5 flasks (2500 cm2)	Flasks scaled linearly and treated as subcomponents
OKT3	150ug	≤180ug at max scale
IL2	High dose	High dose
Number of flasks	1-5	1-4
Harvest Volume reduction	Closed 10:1	Closed 10:1
Concentrate/Wash	LOVO 100:1	LOVO 1000:1
Formulation	1:1 CS10 (5% DMSO)	1:1 CS10 (5% DMSO)
Shipment	Vapor phase LN	Vapor phase LN
Infusion	Thawed IV gravity	Thawed IV gravity

Figure 66

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 10-17 Mycoplasma
Day 10-17 Endotoxin
Day 10-17 Phenotyping
Day 10-17 TVC count
Day 10-17 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
Lymphodepletion
Surgery
Culture Initiation
In-Process Testing
(Visual inspection, Sterility, Myco, Phenotype, IFNg)
Ship
IFN-g results
Final Sterility results
Infuse
Harvest
Sterility, TVC, Viability, Mycoplasma, Phenotype, Gram Stain, Endotoxin, IFN-g)
TIL Activation
Scale Up
0
7
11
17
21
24
* Restimulation for ELISA on Day 10-11
will be from a fresh / frozen in-process sample reportable
On Day 14

Figure 67

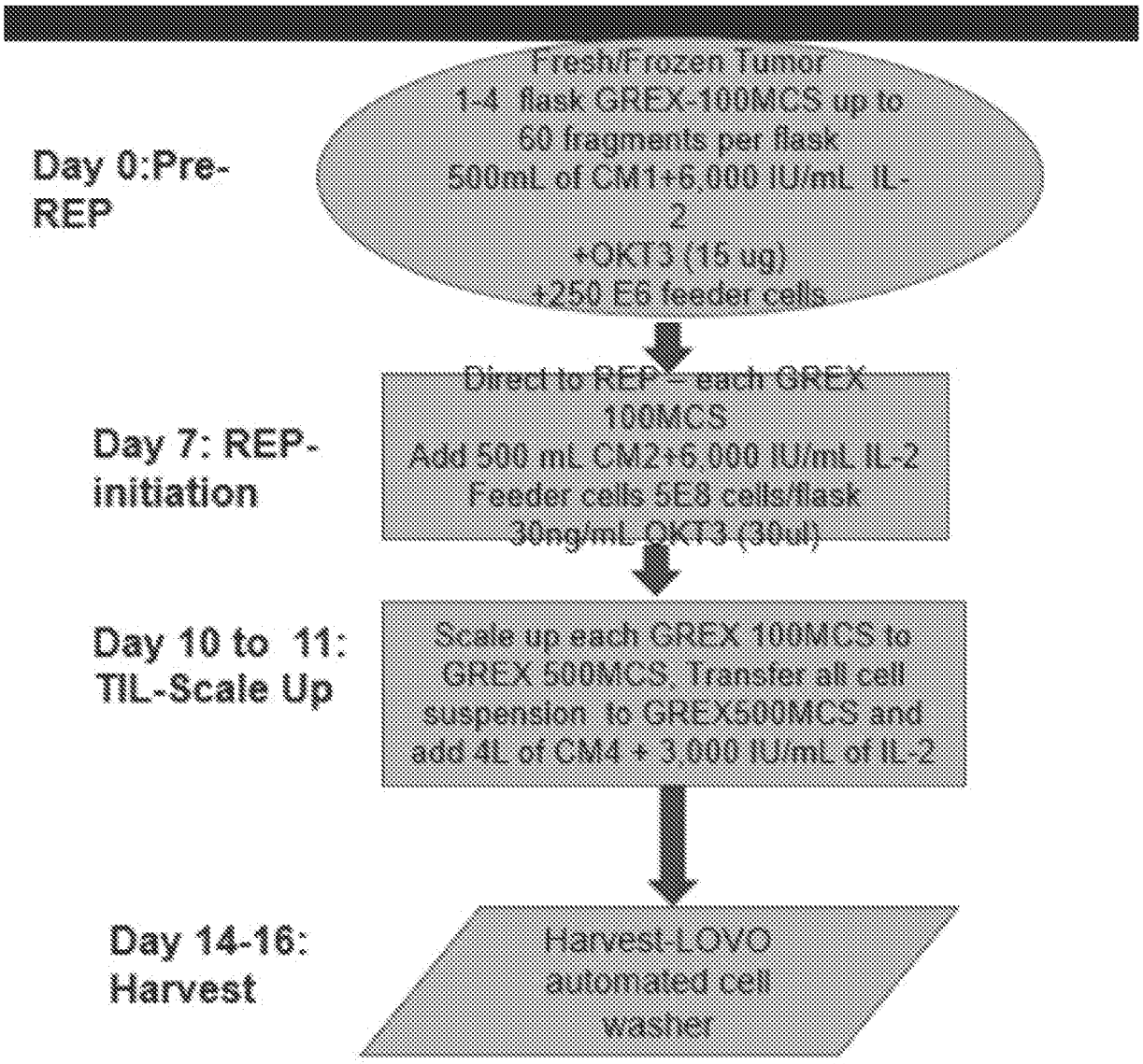

Day 0:Pre-REP
Fresh/Frozen Tumor
1-4 flask GREX-100MCS up to 60 fragments per flask
500mL of CM1+6,000 IU/mL IL-2
+OKT3 (15 ug)
+250 E6 feeder cells
Day 7: REP-initiation
Direct to REP – each GREX 100MCS
Add 500 mL CM2+6,000 IU/mL IL-2
Feeder cells 5E8 cells/flask
30ng/mL OKT3 (30ul)
Day 10 to 11: TIL-Scale 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 68

Data Summary of testing Day 16/17

Testing Parameter	1st Eng. Run Day 17 10 March 2019	2nd Eng. Run Day 17 11 March 2019	3rd Eng. Run Day 17 25 March 2019	Gen 2 (n=47)		
				Mean	Median	Stdev
Pre-LOVO TVC	17.3E+09	46.1E+09				
Pre-LOVO % Viability	90.5 %	94.3%				
Post-LOVO TVC	17.9E+09	59.4E+09	48.2E+09			
Post-LOVO % Viability	85.3%	90.7%	89%			
TVC Post LOVO % Recovery	103.5%	128.9%				
%CD45+/CD3+	88.0%	98.3%				
IFNγ (pg/mL)	11,618	19,235		10,005	9,637	5,128
GrzB (pg/mL)	11,326	27,441		19,929	16,040	
Sterility	Pending (*)	Pending (*)				
Endotoxin (total EU units)	154.2 EU	195.9 EU				
Mycoplasma	Negative	Negative				
Gram Staining	No organisms detected	No organisms detected				

Figure 69

**Adds up to entire TIL sample (100% Live, CD14-, except for monocytes)*

Characteristic	Eng run #1	Eng run #2
NK cells (CD3-CD56+) [%]	10.9	1.5
B cells (CD3-CD19+) [%]	0.2	0.2
Monocytes (CD14+) [%]	0.0	0.0
TCRαβ [%]	87.1	96.3
TCRγδ [%]	1.2	1.6
Sum	99.4	99.6

**Adds up to 100% TCRαb*

Characteristic	Eng run #1	Eng run #2
Naive: CCR7+CD45RA+ [%]	0.0	0.1
T-EM: CCR7-CD45RA- [%]	92.2	84.4
T-CM: CCR7+CD45RA- [%]	7.7	15.5
TEff/TEMRA: CCR7-CD45RA+ [%]	0.1	0.1
Sum	100.0	100.0

**Adds up to 100% Live, CD14-*

Characteristic	Eng run #1	Eng run #2
TCRαβ+ CD4+ [%]	89.6	71.8
TCRαβ+ CD8+ [%]	10.0	27.8
Sum	99.6	99.6

Figure 70

**Adds up to entire TIL sample (100% Live, CD14-, except for monocytes)*

Characteristic	PD run #1 L4063	PD run #2 L4064
NK cells (CD3-CD56+) [%]	1.04	0.52
B cells (CD3-CD19+) [%]	0.0076	0.013
Monocytes (CD14+) [%]	0.032	0.043
TCRαβ [%]	95.5	96.7
TCRγδ [%]	2	1.86
Sum	98.6	99.14

**Adds up to 100% TCRab*

Characteristic	PD run #1 L4063	PD run #2 L4064
Naive: CCR7+CD45RA+ [%]	0.36	0.07
T-EM: CCR7-CD45RA-[%]	97.4	96.7
T-CM: CCR7+CD45RA-[%]	1.39	2.78
T-EFF/TEMRA: CCR7-CD45RA+[%]	0.88	0.44
Sum	100.0	100.0

Characteristic	PD run #1 L4063	PD run #2 L4064
TCRαβ+ CD4+ [%]	77.5	57.4
TCRαβ+ CD8+ [%]	16.8	36.1
Sum	94.3	93.5

Figure 71

Characteristic	OV-8025			OV-8026			OV-8028			OV-8022		
	FRER*	Gen 2^	Gen 3.1	FRER*	Gen 2	Gen 3.1	FRER	Gen 2^	Gen 3.1	FRER	Gen 2^	Gen 3.1*
Purity												
T cells (CD45+CD3+) (%)	-	-	99.2	-	75.1	97.5	97	-	97.6	53.6	16.4	-
NK cells (CD3-CD56+) (%)	-	-	0.2	-	8.4	1.1	0.4	-	0.1	44.6	81.1	-
B cells (CD3-CD19+) (%)	-	-	0	-	2.1	0	0	-	0	1.3	3.3	-
Monocytes (CD14+) (%)	-	-	0	-	0.2	0	0	-	0	2	0.1	-
Identity: T cells												
TCR αβ (%)	-	-	99.8	-	71.9	96.4	98.7	-	99.4	32.2	12.4	-
TCR γδ (%)	-	-	0.3	-	0.3	0.6	0	-	0.1	6.1	0	-
CD3+ CD4+ (%)	-	-	85.7	-	63.1	81.5	81.6	-	92.3	77.9	48.6	-
CD3+ CD8+ (%)	-	-	12	-	23	14.4	15.5	-	5.2	16.6	25.1	-
Memory Phenotype-TCRαβ+												
Naive: CCR7+CD45RA+ (%)	-	-	0	-	0.4	0	2.1	-	0	0	0.1	-
TEM: CCR7-CD45RA- (%)	-	-	98.5	-	88.7	97.5	92.6	-	97.1	89.1	93.8	-
TCM: CCR7+CD45RA- (%)	-	-	1	-	7.5	0.6	1.9	-	2.5	0.1	0	-
TEFF/TEMRA: CCR7-CD45RA+(%)	-	-	0.4	-	3.4	1.8	2.4	-	0.3	10.8	6.1	-

FR ER, Frozen tumor, Early REP
*, Condition not tested
^, Sampling issue, low TVC count or non-viable cells on thawing

Figure 72

Characteristic	Hierarchy	Eng run #1	Eng run #2
NK cells (CD3-CD56+) [%]	S1/S2/All/Live/CD14-/CD3-/NK | Freq. of null (%)	10.9	1.5
B cells (CD3-CD19+) [%]	S1/S2/All/Live/CD14-/CD19 | Freq. of null (%)	0.2	0.2
Monocytes (CD14+) [%]	S1/S2/All/Live/CD14+ | Freq. of Parent (%)	0.0	0.0
TCRab [%]	S1/S2/All/Live/CD14-/TCRab | Freq. of null (%)	87.1	96.3
TCRgd [%]	S1/S2/All/Live/CD14-/TCRgd | Freq. of null (%)	1.2	1.6
TCRab CD4 + [%]	S1/S2/All/Live/CD14-/TCRab/CD4 | Freq. of Parent (%)	88.6	71.8
TCRab CD8+ [%]	S1/S2/All/Live/CD14-/TCRab/CD8 | Freq. of Parent (%)	10.0	27.8
Naive CD4+ [%]	S1/S2/All/Live/CD14-/TCRab/Q2: CCR7+, CD45RA+ | Freq. of Parent (%)	0.0	0.1
TEMRA CD4+ [%]	S1/S2/All/Live/CD14-/TCRab/Q4: CCR7-, CD45RA- | Freq. of Parent (%)	92.2	84.4
TCM CD4+ [%]	S1/S2/All/Live/CD14-/TCRab/Q3: CCR7+, CD45RA- | Freq. of Parent (%)	7.7	15.5
TEM CD4+ [%]	S1/S2/All/Live/CD14-/TCRab/Q1: CCR7-, CD45RA+ | Freq. of Parent (%)	0.1	0.1
Naive CD8+ [%]	S1/S2/All/Live/CD14-/TCRab/Q6: CD62L+, CD45RA+ | Freq. of Parent (%)	0.2	0.1
TEM CD8+ [%]	S1/S2/All/Live/CD14-/TCRab/Q8: CD62L-, CD45RA- | Freq. of Parent (%)	47.7	49.5
TCM CD8+ [%]	S1/S2/All/Live/CD14-/TCRab/Q7: CD62L+, CD45RA- | Freq. of Parent (%)	52.1	50.4
TEMRA CD8+ [%]	S1/S2/All/Live/CD14-/TCRab/Q5: CD62L-, CD45RA+ | Freq. of Parent (%)	0.1	0.0

**Adds up to CD3+CD45+ %*

Experiment	Eng run #1	Eng run #2
TCRgd [%]	87.1	96.3
TCRab [%]	1.2	1.6
Sum	88.3	97.9

Figure 73

Characteristic	Hierarchy	CD4+		CD8+	
		Eng run #1	Eng run #2	Eng run #1	Eng run #2
CD27+ (%)	S1/S2/All/Live/CD14-/TCRab/CD4 or CD8/CD44+/8+CD27+ | Freq. of Parent (%)	0.7	2.0	2.1	12.7
CD28+ (%)	S1/S2/All/Live/CD14-/TCRab/CD4 or CD8/CD44+/8+CD28+ | Freq. of Parent (%)	41.2	50.1	3.6	13.9
CD57+ (%)	S1/S2/All/Live/CD14-/TCRab/CD4/CD44+/8+CD57+ | Freq. of Parent (%)	1.0	6.9	0.1	3.7
2B4+ (%)	S/S/Live/CD3/CD4 or CD8/btla | Freq. of Parent (%)	4.5	5.5	69.1	55.7
BTLA+ (%)	S/S/Live/CD3/CD4 or CD8/2b4 | Freq. of Parent (%)	67.5	86.1	79.6	89.1
CCR4+ (%)	S/S/Live/CD3/CD4 or CD8/CCR4+ | Freq. of Parent (%)	94.1	93.6	84.2	96.6
CD25+ (%)	S/S/Live/CD3/CD4 or CD8/CD25 | Freq. of Parent (%)	63.1	61.0	47.5	51.8
CD69+ (%)	S/S/Live/CD3/CD4 or CD8/CD69 | Freq. of Parent (%)	47.0	22.8	45.4	25.8
CD95+ (%)	S/S/Live/CD3/CD4 or CD8/CD95 | Freq. of Parent (%)	99.2	98.1	99.1	97.6
CD103+ (%)	S/S/Live/CD3/CD4 or CD8/CD103 | Freq. of Parent (%)	4.2	10.7	25.0	36.9
CXCR3+ (%)	S/S/Live/CD3/CD4 or CD8/CXCR3 | Freq. of Parent (%)	58.1	76.7	89.3	88.3
KLRG1+ (%)	S/S/Live/CD3/CD4 or CD8/KLRG1 | Freq. of Parent (%)	0.3	8.9	3.1	35.8
LAG3+ (%)	S/S/Live/CD3/CD4 or CD8/Lag3 | Freq. of Parent (%)	5.0	3.3	19.4	21.2
PD1+ (%)	S/S/Live/CD3/CD4 or CD8/PD1 | Freq. of Parent (%)	29.3	22.1	11.2	7.2
TIGIT+ (%)	S/S/Live/CD3/CD4 or CD8/Tigit | Freq. of Parent (%)	68.1	53.2	74.9	65.8
TIM3+ (%)	S/S/Live/CD3/CD4 or CD8/TIM3 | Freq. of Parent (%)	93.6	81.8	91.2	84.4

Figure 74

	1:100 Stim/1:50 Unstim			1:200 Stim/1:100 Unstim			1:400 Stim/1:200 Unstim					
Sample	1	2	3	4	5	6	7	8	9	Avg	Stim-Unstim	Fold Induction
Eng 1 Stimulated	13001.49	16033.00	14083.24	11006.85	15254.59	12534.24	9985.83	14257.22	11669.82	13089.81	11325.97	7.42
Eng 1 Unstimulated	2155.31	2445.64	2108.37	1727.43	1932.96	1566.52	1119.057	1611.132	1158.179	1763.84		
Eng 1 Stimulated Ctrl	36491.92	31766.20	31601.13	26385.05	30852.42	29752.34	26175.43	33462.43	29728.52	29579.45	13486.52	1.84
Eng 1 Unstimulated Ctrl	16209.90	16478.69	16936.51	15659.69	16385.60	17083.36	15419.07	15683.49	14980.40	16092.97		

	1:100 Stim/1:50 Unstim			1:200 Stim/1:100 Unstim			1:400 Stim/1:200 Unstim					
Sample	1	2	3	4	5	6	7	8	9	Avg	Stim-Unstim	Fold Induction
Eng 2 Stimulated	31509.54	30435.33	33822.61	27299.28	29091.87	29432.13	29027.45	30685.94	31138.70	30271.45	27441.29	10.70
Eng 2 Unstimulated	3236.50	3442.58	3830.97	2754.12	2839.66	2892.96	2047.795	2291.994	2324.838	2830.16		
Eng 2 Stimulated Ctrl	21515.93	20346.31	19425.54	16239.25	17398.91	18131.38	15825.27	18412.15	18278.94	18959.30	6189.32	1.48
Eng 2 Unstimulated Ctrl	13716.23	13445.80	13563.69	12782.28	12678.37	13122.55	11888.75	11675.48	12046.29	12769.97		

Figure 75A

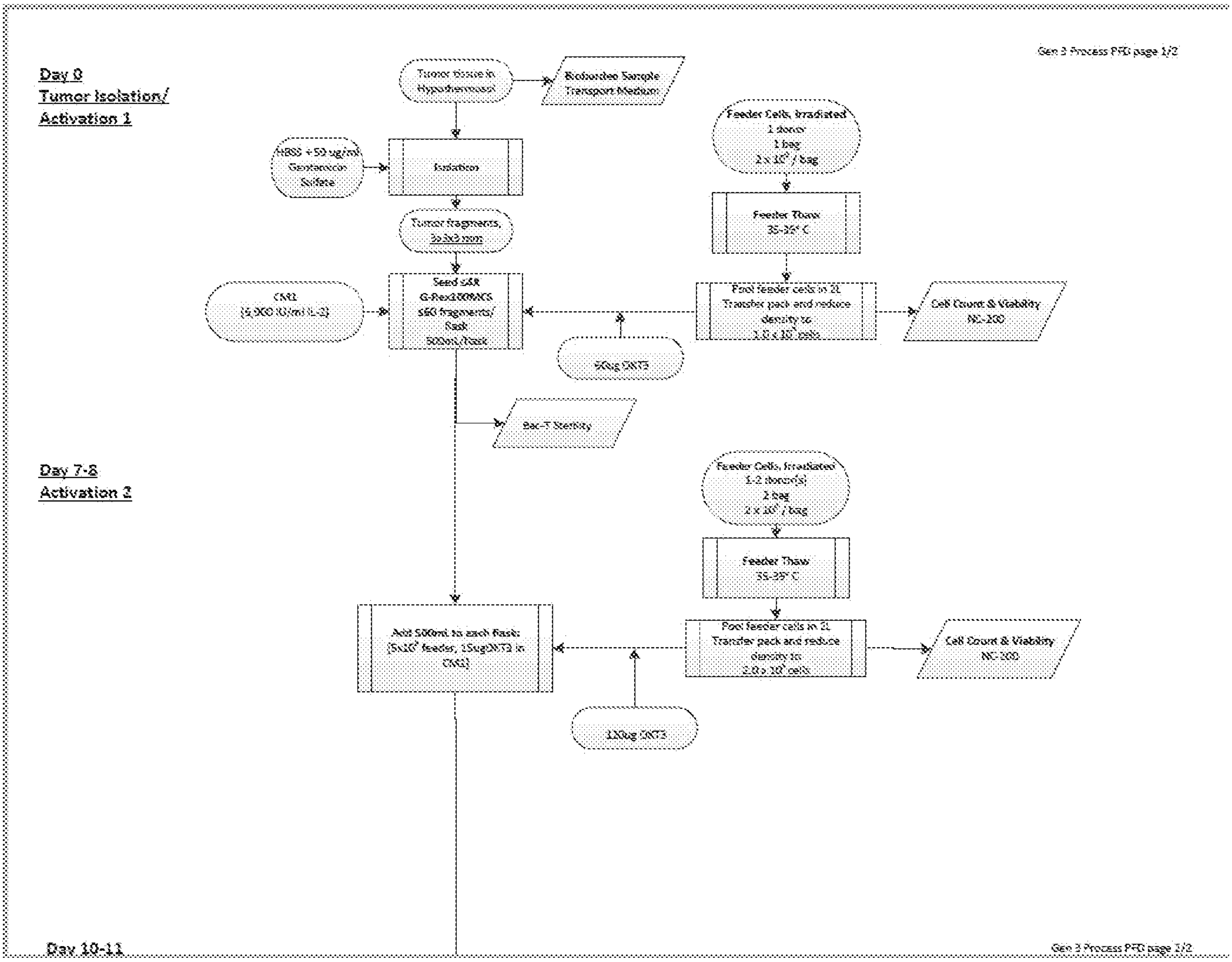

Gen 3 Process PFD page 1/2
Day 0
Tumor Isolation/
Activation 1
Tumor tissue in Hypothermosol
Bioburden Sample Transport Medium
HBSS + 50 ug/ml Gentamicin Sulfate
Isolation
Tumor fragments, 3x3x3 mm
Feeder Cells, Irradiated
1 donor
1 bag
Feeder Thaw
Pool feeder cells in 2L Transfer pack and reduce density to
Cell Count & Viability NC-200
Seed ≤4X G-Rex100MCS ≤60 fragments/ flask 500mL/flask
Bac-T Sterility
Day 7-8
Activation 2
Feeder Cells, Irradiated
1-2 donor(s)
Feeder Thaw
Pool feeder cells in 2L Transfer pack and reduce density to
Cell Count & Viability NC-200
Add 500ml to each flask:
Day 10-11
Gen 3 Process PFD page 1/2

Figure 75B

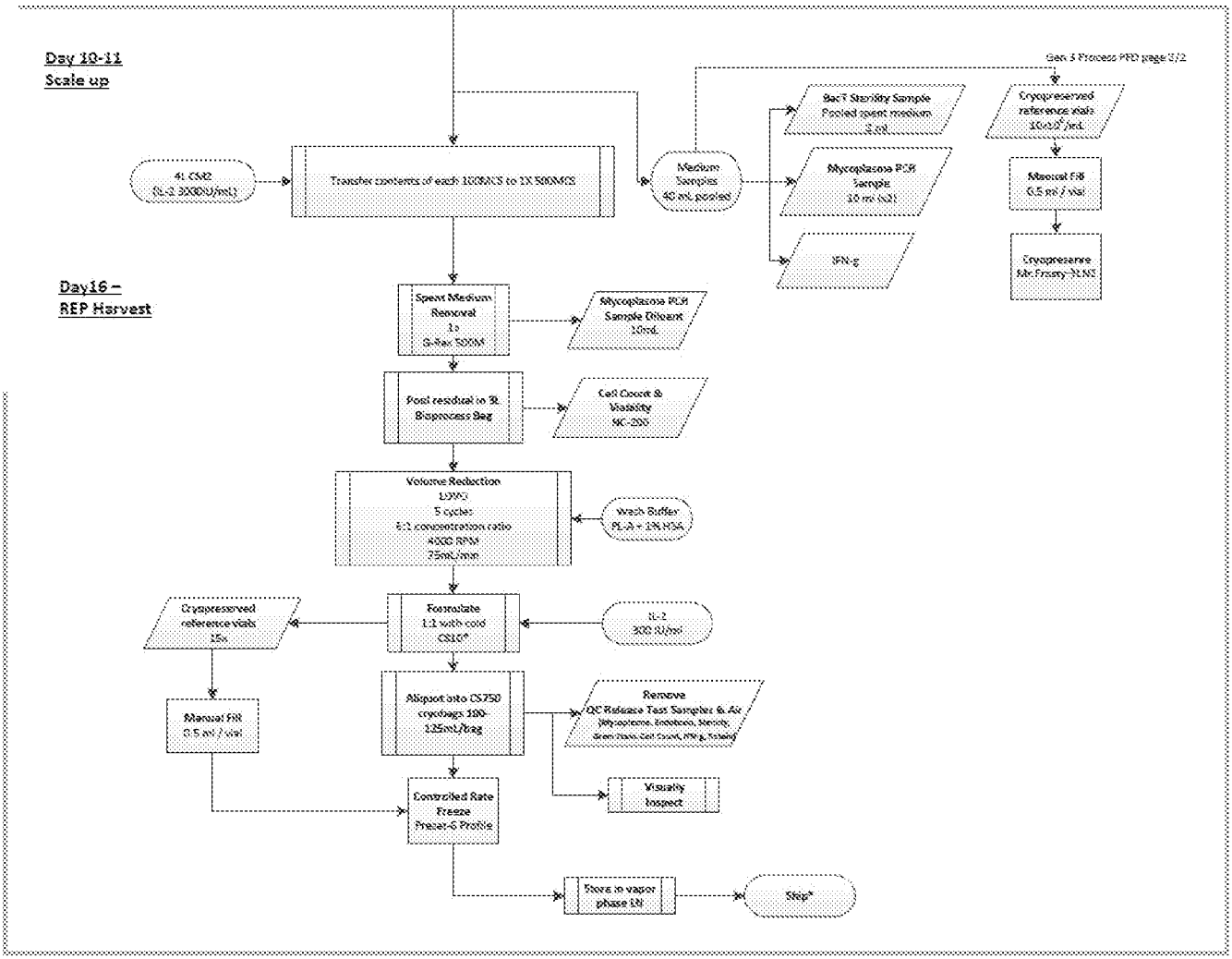

Day 10-11
Scale up
Gen 3 Process PFD page 2/2
4L CS02
IL-2 3000IU/mL
Transfer contents of each 1000NUS to 1X 500MCS
Medium Samples
40 mL pooled
BacT Sterility Sample
Pooled spent medium
3 mL
Mycoplasma PCR Sample
10 mL (x2)
IFN-g
Cryopreserved reference vials
10x2.0^7/mL
Mascot Fill
0.5 mL / vial
Cryopreserve Mr Frosty -80 NS
Day16 –
REP Harvest
Spent Medium Removal
1x
G-Rex 500M
Mycoplasma PCR Sample Diluent
10mL
Post residual to 3L Bioprocess Bag
Cell Count & Viability
NC-200
Volume Reduction
LOVO
5 cycles
5:1 concentration ratio
4000 RPM
75mL/min
Wash Buffer
PL-3 + 1% HSA
Cryopreserved reference vials
15x
Formulate
1:1 with cold CS10*
IL-2
3000 IU/ml
Mascot Fill
0.5 mL / vial
Aliquot into CS750 cryobags 100-125mL/bag
Remove
QC Release Test Samples & Air
Mycoplasma, Endotoxin, Sterility, Gram Stain, Cell Count, IFN-g, Potency
Visually Inspect
Controlled Rate Freeze
Prime-S Profile
Store in vapor phase LN
Ship*

Figure 76

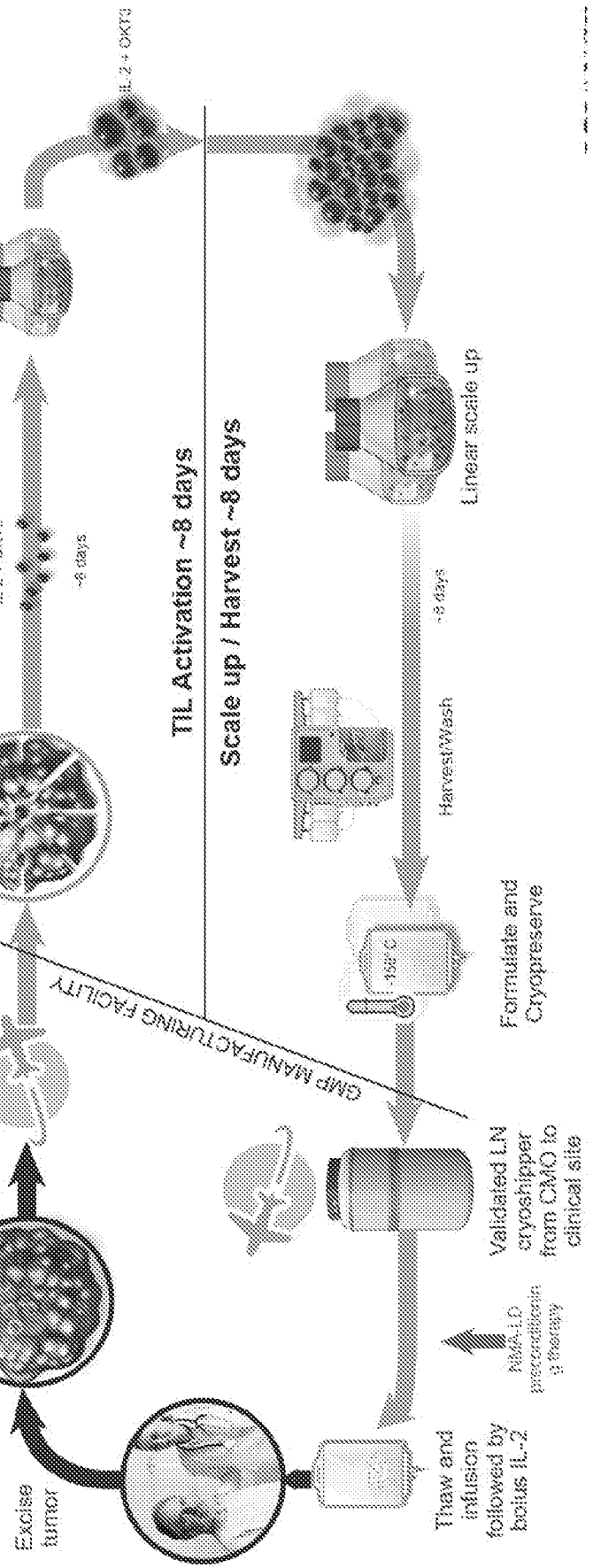

Excise tumor
Express courier from clinical site to CMO
Cut into fragments
TIL culture activation
IL-2 + OKT3
~6 days
TIL Activation ~8 days
Scale up / Harvest ~8 days
GMP MANUFACTURING FACILITY
Harvest/Wash
~8 days
Linear scale up
IL-2 + OKT3
Formulate and Cryopreserve
Validated LN cryoshipper from CMO to clinical site
Thaw and infusion followed by bolus IL-2

Figure 77

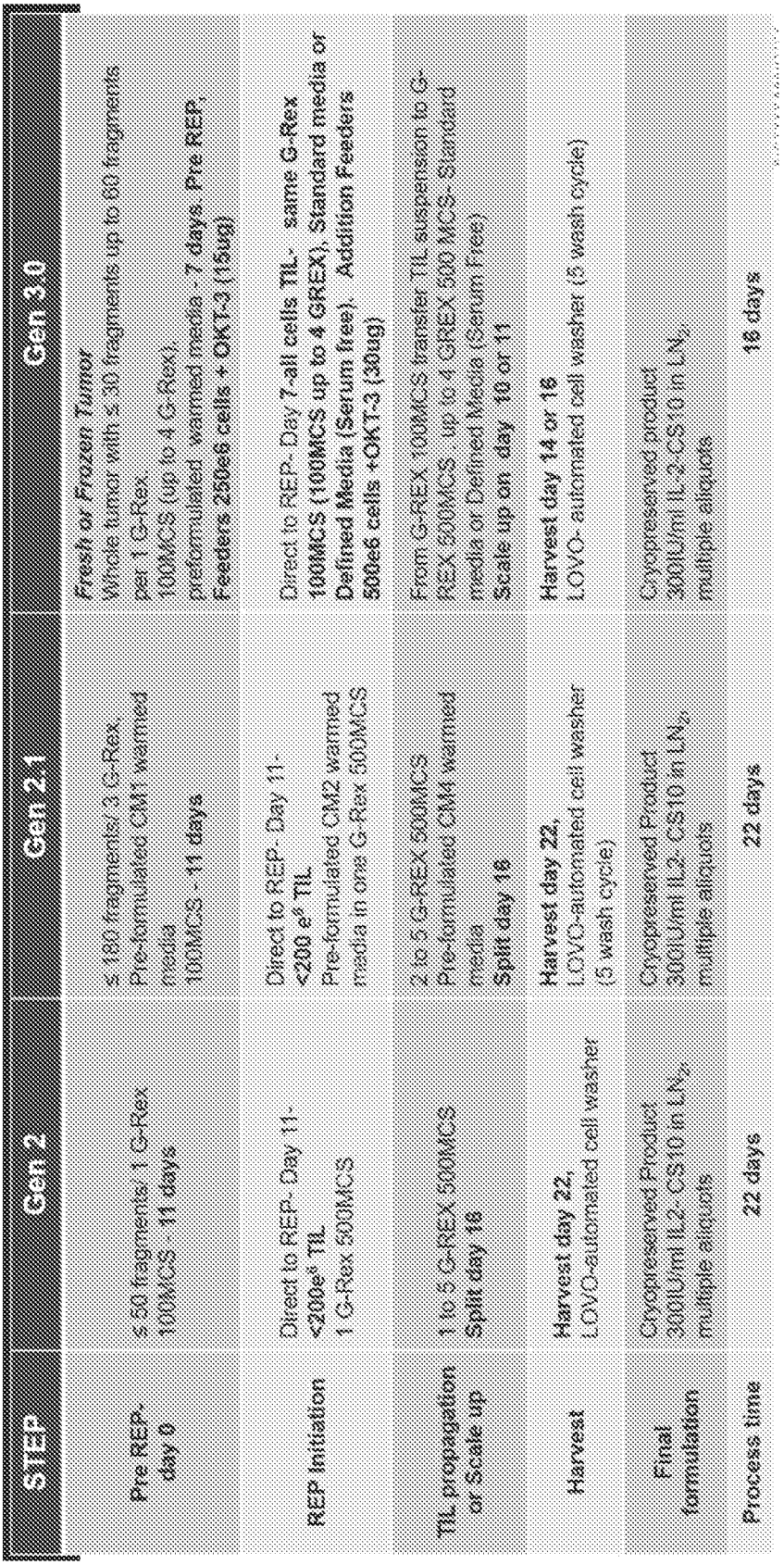

STEP
Gen 2
Gen 2.1
Gen 3.0
Pre REP- day 0
≤ 60 fragments/ 1 G-Rex 100MCS - 11 days
≤ 180 fragments/ 3 G-Rex Pre-formulated CM1 warmed media 180MCS - 11 days
Fresh or Frozen Tumor Whole tumor with ≤ 30 fragments up to 60 fragments per 1 G-Rex 100MCS (up to 4 G-Rex), preformulated, warmed media - 7 days. Pre REP: Feeders 250e6 cells + OKT-3 (15ug)
REP Initiation
Direct to REP- Day 11- <200e6 TIL 1 G-Rex 500MCS
Direct to REP- Day 11- <200 e6 TIL Pre-formulated CM2 warmed media in ons 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 500e6 cells +OKT-3 (30ug)
TIL propagation or Scale up
1 to 5 G-REX 500MCS Split day 18
2 to 5 G-REX 500MCS Pre-formulated CM4 warmed media Split day 18
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 (5 wash cycle)
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 300U/ml IL2- CS10 in LN2, multiple aliquots
Cryopreserved Product 300U/ml IL2- CS10 in LN2, multiple aliquots
Cryopreserved product 300U/ml IL-2-CS10 in LN2, multiple aliquots
Process time
22 days
22 days
16 days

Figure 78

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 doubling 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 79

Table 2. Description of Gen 3 optimization conditions.

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 80

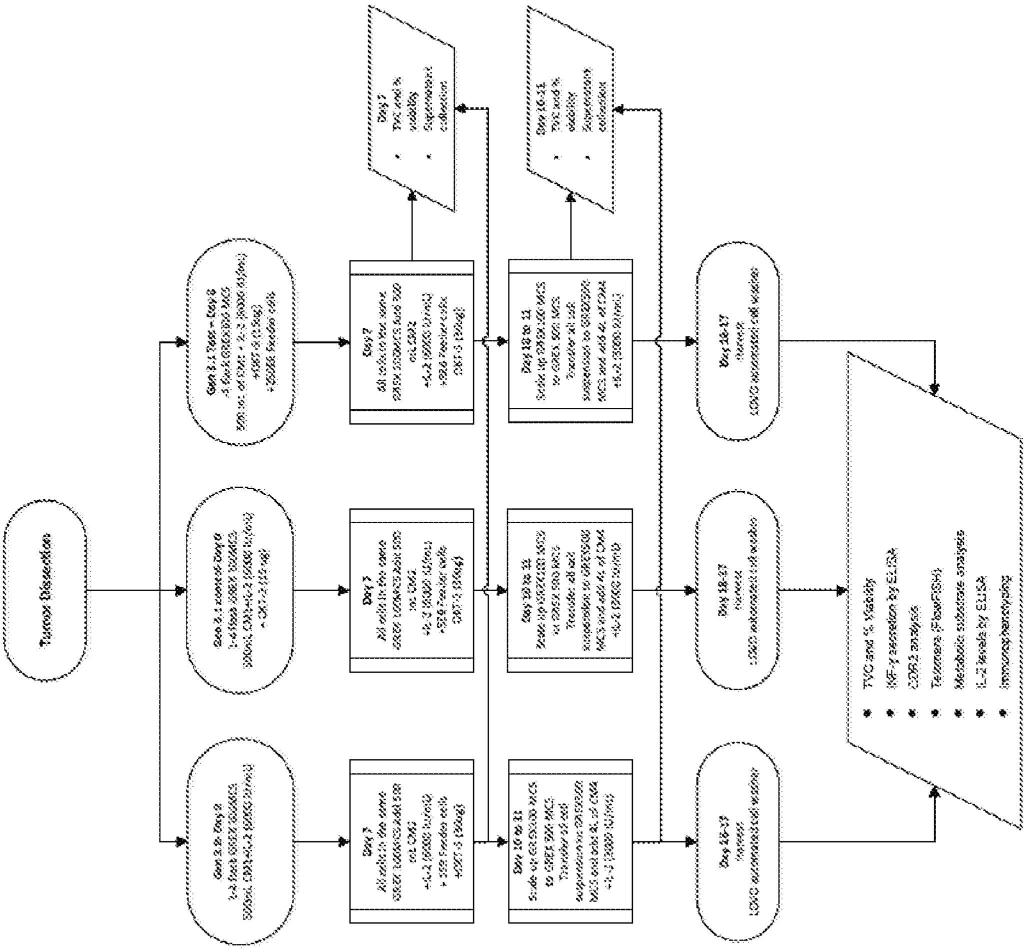


Figure 81

Process Day	L4063 in Standard Media			L4064 in Serum Free Media		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Number of fragments	53	53	53	21	21	21
Average TVC at harvest per fragment	1.37E+08	3.19E+08	3.53E+08	5.90E+08	7.57E+08	9.29E+08
TVC day 7 (*)	2.59E+07	6.43E+07	1.45E+08	7.35E+06	9.28E+07	1.39E+08
TVC day 10/11 (*)	6.07E+08	1.66E+09	1.83E+09	7.48E+08	1.28E+09	1.72E+09
TVC harvest day 16/17	7.26E+09	1.69E+10	1.87E+10	1.24E+10	1.59E+10	1.95E+10
Fold Expansion (day 10 or 11 /day 7)	23.4	25.8	12.6	101.8	13.8	12.4
Fold Expansion (Harvest day 16 / day 7)	280.3	262.8	129.0	1687.1	171.3	140.3

(*) Cell counts for Day 7 and Day 10/11 were taken FIO. Based on the design of the process, samples were pulled directly from each flask after swirling its contents without volume reduction. As such, analysis and conclusions of cell count data from these samples is limited by the nature of the sampling methodology.

Figure 82

Process Day	L4063 in Standard media			L4064 in CTS Serum Free media		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	74.6%	92.1%	88.7%	88.0%	92.8%	95.0%
Scale up	92.1%	95.4%	94.0%	90.4%	94.3%	95.1%
Harvest	79.5%	89.4%	94.8%	88.8%	81.9%	84.9%

Figure 83

Tumor ID	L4063 in Standard media			L4064 in CTS Serum Free media		
Markers	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
L/D Aqua	82.2	89.4	91.0	76.6	83.1	89.5
NK	1.3	0.4	1.0	2.5	0.2	0.5
TCRa/b+	88.6	97.5	95.5	93.0	97.3	96.7
CD4	56.4	85.8	77.5	33.8	64.8	57.4
CD4/CD27	3.8	2.1	3.4	6.7	0.7	2.2
CD4/CD28	82.7	71.8	80.2	97.5	73.5	66.8
CD4/CD56	0.4	0.5	0.7	0.5	0.5	0.3
CD4/CD57	6.7	17.0	20.5	16.0	7.6	8.0
CD8	29.6	11.2	16.8	51.9	28.9	36.1
CD8/CD27	15.0	25.6	30.5	14.1	3.3	6.7
CD8/CD28	47.5	23.0	24.5	88.0	45.1	37.3
CD8/CD56	3.3	2.3	4.2	6.6	10.3	10.0
CD8/CD57	0.3	1.2	1.1	1.3	2.0	2.5

Figure 84

Tumor ID	L4063 in Standard media			L4064 in CTS Serum Free media		
Markers	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
CD3/CD4	56.4	85.8	77.5	33.8	64.8	57.4
TEMRA of CD4	0.4	0.7	0.4	1.1	0.7	0.2
NAIVE of CD4	0.3	0.2	0.2	0.5	0.1	0.0
CM of CD4	45.6	16.8	29.3	46.3	30.6	29.9
EM of CD4	53.7	82.3	70.2	52.1	68.6	69.9
CD3/CD8	29.6	11.2	16.8	51.9	28.9	36.1
TEMRA of CD8	3.1	0.5	0.9	6.1	8.0	5.4
NAIVE of CD8	0.7	0.1	0.4	2.2	0.7	1.0
CM of CD8	22.0	15.8	29.7	61.9	18.9	18.2
EM of CD8	74.3	83.5	69.0	29.8	72.4	75.5

Figure 85

Tumor ID	L4063 in Standard media			L4064 in CTS Serum Free media		
Markers	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
2B4+	2.1	0.6	1.0	2.0	1.2	1.3
BTLA4	99.7	95.4	96.9	99.5	98.8	98.1
CD25+	48.6	10.5	3.9	58.2	41.7	32.1
CD69+	27.1	37.7	21.5	39.4	35.6	30.5
CD95+	99.9	99.9	99.5	99.6	99.0	97.3
CD103+	0.7	1.2	0.4	1.4	1.3	1.2
KLRG1+	1.6	3.7	3.3	11.4	8.3	10.7
LAG3+	5.6	8.2	3.3	1.5	1.6	1.4
PD1+	77.4	58.4	41.6	34.1	28.4	26.7
TIGIT+	68.5	40.3	30.3	45.8	52.1	52.2
TIM3+	58.0	42.4	59.4	61.0	58.4	56.8

Figure 86

Tumor ID	L4063 in Standard media			L4064 in CTS Serum Free media		
Markers	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
2B4+	65.8	56.6	70.5	13.6	21.5	20.7
BTLA4	99.5	96.6	98.2	99.3	97.4	96.1
CD25+	41.4	10.5	5.9	60.6	32.0	17.1
CD69+	44.9	28.0	20.0	33.2	30.6	22.7
CD95+	99.6	99.6	98.9	98.9	96.1	93.5
CD103+	29.4	27.9	24.5	10.9	6.0	7.3
CXCR3+	99.9	99.9	99.8	99.3	99.2	99.2
KLRG1+	7.1	4.6	6.7	13.2	14.5	14.1
LAG3+	28.6	22.5	11.9	2.8	1.6	1.4
PD1+	56.9	54.5	51.7	25.4	20.0	23.6
TIGIT+	90.8	93.7	93.3	92.1	90.4	90.5
TIM3+	66.7	49.1	58.6	65.6	46.8	43.6

Figure 87

TIL Characterization	L4063 in Standard media			L4064 in CTS Serum Free media		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
IFNγ (pg/mL)	907	428	2863	1485	3130	3863

Results are reported as mean of triplicate samples from one representative experiment

Figure 88

Process Day	L4063 in Standard media			L4064 in CTS Serum Free media		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	12319.6	11928.7	11786.7	5891.6	5671.8	389.4[1]
Scale Up	32095.1	30368.6	26145.0	*	*	*
Harvest	17309.3	13828.4	16019.2	656.0	826.2	768.9

Results are reported as mean of triplicate samples from one representative experiment

(*) Spent media from day 10 /11 on L4064 was not collected during the execution of the runs.

(1) The data for Gen 3.1 Test L4064 on reactivation day was significantly below the corresponding values for Gen 3.1 control and Gen 3.0 conditions. Due to the low amount of sample taken, it was not possible to repeat the assay to verify the accuracy of this number.

Figure 89

Glucose (g/L)						
	L4063 in Standard media			L4064 in CTS Serum Free media		
Process Day	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	1.8	1.7	1.4	4.3	4.1	3.8
Scale Up	1.4	0.9	0.8	*	*	*
Harvest	1.5	0.9	1.0	2.8	2.1	2.2

(*) Spent media from day 10 /11 on L4064 was not collected during the execution of the runs.

Figure 90

Lactate (g/L)						
	L4063 in Standard media			L4064 in CTS Serum Free media		
Process Day	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	0.2	0.2	0.5	0.1	0.2	0.5
Scale Up	0.7	1.1	1.2	*	*	*
Harvest	1.1	1.7	1.6	1.5	2.0	1.9

(*) Spent media from day 10 /11 on L4064 was not collected during the execution of the runs.

Figure 91

Glutamine (mmol/L)						
	L4063 in Standard media			L4064 in CTS Serum Free media		
Process Day	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	2.0	2.0	2.0	1.3	1.2	1.1
Scale Up	1.7	1.1	1.1	*	*	*
Harvest	1.9	1.3	1.3	0.7	0.6	0.6

(*) Spent media from day 10 /11 on L4064 was not collected during the execution of the runs.

Figure 92

Glutamax (mmol/L)						
	L4063 in Standard media			L4064 in CTS Serum Free media		
Process Day	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	2.2	2.2	2.2	1.4	1.3	1.2
Scale Up	1.9	1.2	1.2	*	*	*
Harvest	2.1	1.5	1.5	0.8	0.7	0.7

(*) Spent media from day 10 /11 on L4064 was not collected during the execution of the runs.

Figure 93

Ammonia (mmol/L)						
	L4063 in Standard media			L4064 in CTS Serum Free media		
Process Day	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Reactivation	1.9	1.9	2.0	0.7	0.8	0.9
Scale Up	1.8	2.2	2.3	*	*	*
Harvest	2.1	2.5	2.5	1.0	1.0	0.9

(*) Spent media from day 10 /11 on L4064 was not collected during the execution of the runs.

Figure 94

TIL Characterization	L4063 in Standard media			L4064 in CTS Serum Free media		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Relative Telomere Length	7.7	6.3	6.1	6.4	6.7	6.6

Figure 95

TIL Characterization	L4063 in Standard media			L4064 in CTS Serum Free media		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
uCD3	14005.0	18149.0	17181.0	15107.0	21460.0	21503.0
Shanon Entropy Index	5.9	10.8	10.5	8.5	10.7	10.4

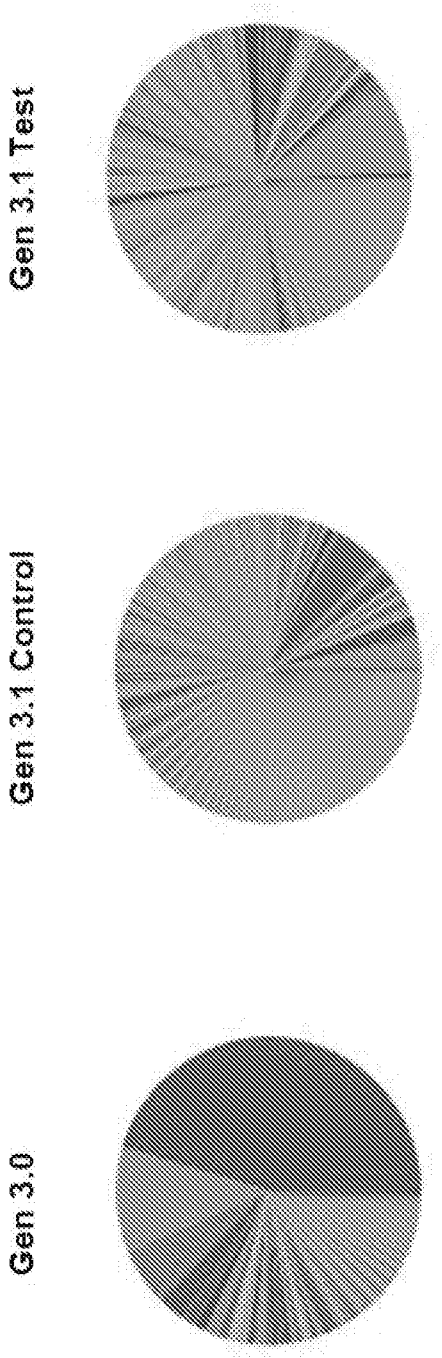


Figure 96

Figure 97

Number of u CDR3 (%overlap) L4063	All uCDR3's			Top 80% uCDR3's		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Gen 3.0	14005	3240 (23%)	2959 (21%)	1104	1000 (90 %)	975 (88%)
Gen 3.1 Control	-	18149	-	-	2437	-
Gen 3.1 Test	-	-	17181	-	-	2331

Figure 98

Number of u CDR3 (%overlap) L4064	All uCDR3's			Top 80% uCDR3's		
	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test	Gen 3.0	Gen 3.1 Control	Gen 3.1 Test
Gen 3.0	15107	5486 (36%)	5541 (36%)	2478	2186 (88%)	2163 (87%)
Gen 3.1 Control	-	21460	-	-	3246	-
Gen 3.1 Test	-	-	21503	-	-	3263

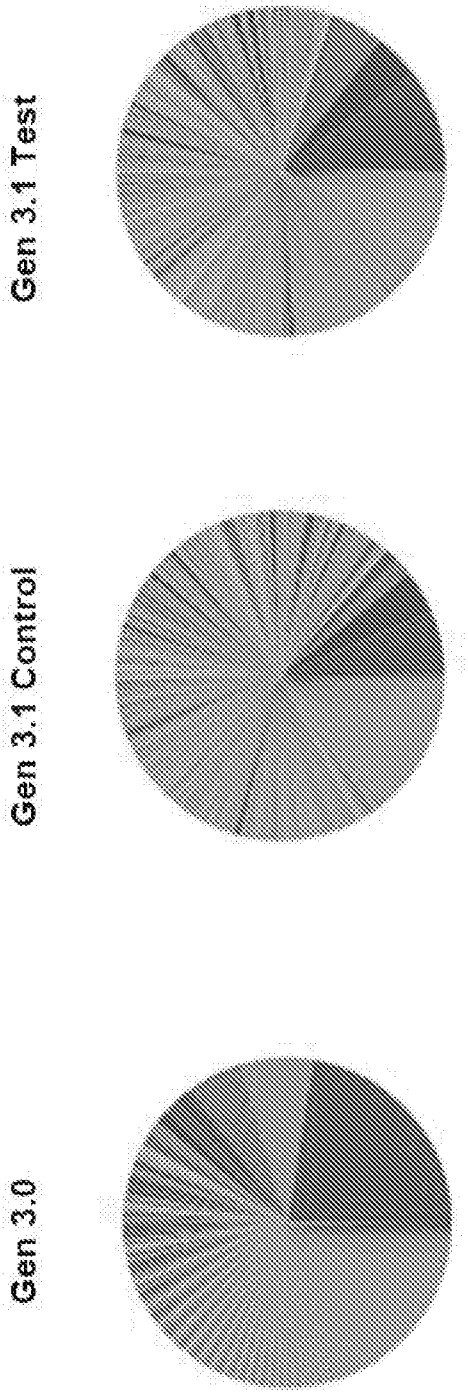


Figure 99

Figure 100

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 101

Test	Acceptance Criteria	Gen 3.1Test 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 102

Cell counts reactivation Day

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3.0 (A) L4063	500	Total	7.10E+04	6.81E+04		6.96E+04	3.48E+07
		Live	5.27E+04	5.09E+04		5.18E+04	2.59E+07
		Dead	1.83E+04	1.72E+04		1.78E+04	8.88E+06
		% Viability	74.30%	74.80%		74.55%	
Gen 3.1 Control L4063	500	Total	1.41E+05	1.38E+05		1.40E+05	6.98E+07
		Live	1.31E+05	1.26E+05		1.29E+05	6.43E+07
		Dead	9.84E+03	1.21E+04		1.10E+04	5.49E+06
		% Viability	93.00%	91.20%		92.10%	
Gen 3.1 Test L4063	500	Total	3.26E+05	3.28E+05		3.27E+05	1.64E+08
		Live	2.83E+05	2.96E+05		2.90E+05	1.45E+08
		Dead	4.25E+04	3.16E+04		3.71E+04	1.85E+07
		% Viability	87.00%	90.30%		88.65%	
Gen 3.0 (A) L4064	500	Total	1.59E+04	1.75E+04		1.67E+04	8.35E+06
		Live	1.39E+04	1.55E+04		1.47E+04	7.35E+06
		Dead	1.98E+03	2.02E+03		2.00E+03	1.00E+06
		% Viability	87.50%	88.50%		88.00%	
Gen 3.1 Control L4064	500	Total	2.02E+05	1.98E+05		2.00E+05	1.00E+08
		Live	1.88E+05	1.83E+05		1.86E+05	9.28E+07
		Dead	1.38E+04	1.51E+04		1.45E+04	7.23E+06
		% Viability	93.20%	92.40%		92.80%	
Gen 3.1 Test L4064	500	Total	2.70E+05	3.15E+05		2.93E+05	1.46E+08
		Live	2.55E+05	3.01E+05		2.78E+05	1.39E+08
		Dead	1.51E+04	1.38E+04		1.45E+04	7.23E+06
		% Viability	94.40%	95.60%		95.00%	

Figure 103

Cell counts Scale Up Day

ID	Volume (mL)		Count 1 (cells/mL)	Count 3 (cells/mL)	Count 2 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3.0 (A) L4063	1000	Total	6.57E+05	6.61E+05		6.59E+05	6.59E+08
		Live	6.00E+05	6.14E+05		6.07E+05	6.07E+08
		Dead	5.71E+04	4.68E+04		5.20E+04	5.20E+07
		% Viability	91.30%	92.90%		92.10%	
Gen 3.1 Control L4063	1000	Total	1.66E+06	1.82E+06		1.74E+06	1.74E+09
		Live	1.59E+06	1.72E+06		1.66E+06	1.66E+09
		Dead	7.17E+04	9.07E+04		8.12E+04	8.12E+07
		% Viability	95.70%	95.00%		95.35%	
Gen 3.1 Test L4063	1000	Total	1.88E+06	2.00E+06		1.94E+06	1.94E+09
		Live	1.76E+06	1.89E+06		1.83E+06	1.83E+09
		Dead	1.20E+05	1.15E+05		1.18E+05	1.18E+08
		% Viability	93.60%	94.30%		93.95%	
Gen 3.0 (A) L4064	1000	Total	7.95E+05	8.58E+05		8.27E+05	8.27E+08
		Live	7.17E+05	7.78E+05		7.48E+05	7.48E+08
		Dead	7.85E+04	8.05E+04		7.95E+04	7.95E+07
		% Viability	90.10%	90.60%		90.35%	
Gen 3.1 Control L4064	1000	Total	1.38E+06	1.32E+06		1.35E+06	1.35E+09
		Live	1.31E+06	1.24E+06		1.28E+06	1.28E+09
		Dead	7.20E+04	8.13E+04		7.67E+04	7.67E+07
		% Viability	94.80%	93.80%		94.30%	
Gen 3.1 Test L4064	1000	Total	1.85E+06	1.76E+06		1.81E+06	1.81E+09
		Live	1.76E+06	1.67E+06		1.72E+06	1.72E+09
		Dead	8.62E+04	8.95E+04		8.79E+04	8.79E+07
		% Viability	95.30%	94.90%		95.10%	

Figure 104

Cell counts Harvest L4063

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3.0 (A) L4063 pre LOVO	602.4	Total	1.68E+07	1.70E+07		1.69E+07	1.02E+10
		Live	1.52E+07	1.54E+07		1.53E+07	9.22E+09
		Dead	1.60E+06	1.65E+06		1.63E+06	9.79E+08
		% Viability	90.50%	90.30%		90.40%	
Gen 3.1 Control L4063 pre LOVO	497.1	Total	3.39E+07	3.90E+07		3.65E+07	1.81E+10
		Live	3.25E+07	3.73E+07		3.49E+07	1.73E+10
		Dead	1.41E+06	1.63E+06		1.52E+06	7.56E+08
		% Viability	95.80%	95.80%		95.80%	
Gen 3.1 Test L4063 pre LOVO	552.3	Total	3.71E+07	3.62E+07		3.67E+07	2.02E+10
		Live	3.56E+07	3.51E+07		3.54E+07	1.95E+10
		Dead	1.50E+06	1.09E+06		1.30E+06	7.15E+08
		% Viability	96.00%	97.00%		96.50%	
Gen 3.0 (A) L4063 post LOVO	165	Total	5.37E+07	5.73E+07	5.50E+07	5.53E+07	9.13E+09
		Live	4.32E+07	4.52E+07	4.36E+07	4.40E+07	7.26E+09
		Dead	1.04E+07	1.21E+07	1.14E+07	1.13E+07	1.86E+09
		% Viability	80.50%	78.80%	79.30%	79.53%	
Gen 3.1 Control L4063 post LOVO	165	Total	1.21E+08	1.01E+08	1.22E+08	1.15E+08	1.89E+10
		Live	1.10E+08	8.85E+07	1.09E+08	1.03E+08	1.69E+10
		Dead	1.11E+07	1.23E+07	1.27E+07	1.20E+07	1.99E+09
		% Viability	90.90%	87.80%	89.60%	89.43%	
Gen 3.1 Test L4063 post LOVO	175	Total	1.05E+08	1.20E+08		1.13E+08	1.97E+10
		Live	9.93E+07	1.14E+08		1.07E+08	1.87E+10
		Dead	5.51E+06	6.14E+06		5.83E+06	1.02E+09
		% Viability	94.70%	94.90%		94.80%	

Figure 105

Cell counts Harvest L4064

ID	Volume (mL)		Count 1 (cells/mL)	Count 2 (cells/mL)	Count 3 (cells/mL)	Average (cells/mL)	Total Cells Average*Volume
Gen 3.0 (A) L4064 pre LOVO	641.3	Total	1.99E+07	1.94E+07		1.97E+07	1.26E+10
		Live	1.83E+07	1.94E+07		1.89E+07	1.21E+10
		Dead	1.60E+06	1.68E+06		1.64E+06	1.05E+09
		% Viability	92.00%	92.00%		92.00%	
Gen 3.1 Control L4064 pre LOVO	493.4	Total	3.98E+07	3.87E+07		3.93E+07	1.94E+10
		Live	3.65E+07	3.55E+07		3.60E+07	1.78E+10
		Dead	3.31E+06	3.22E+06		3.27E+06	1.61E+09
		% Viability	91.70%	91.70%		91.70%	
Gen 3.1 Test L4064 pre LOVO	593.4	Total	3.92E+07	3.79E+07		3.86E+07	2.29E+10
		Live	3.67E+07	3.54E+07		3.61E+07	2.14E+10
		Dead	2.55E+06	2.50E+06		2.53E+06	1.50E+09
		% Viability	93.50%	93.40%		93.45%	
Gen 3.0 (A) L4064 post LOVO	165	Total	8.58E+07	8.34E+07		8.46E+07	1.40E+10
		Live	7.56E+07	7.46E+07		7.51E+07	1.24E+10
		Dead	1.02E+07	8.79E+06		9.50E+06	1.57E+09
		% Viability	88.10%	89.50%		88.80%	
Gen 3.1 Control L4064 post LOVO	165	Total	1.13E+08	1.22E+08	1.15E+08	1.18E+08	1.94E+10
		Live	9.21E+07	1.01E+08	9.45E+07	9.66E+07	1.59E+10
		Dead	2.14E+07	2.13E+07	2.07E+07	2.14E+07	3.52E+09
		% Viability	81.20%	82.60%	82.10%	81.90%	
Gen 3.1 Test L4064 post LOVO	165	Total	1.33E+08	1.45E+08		1.39E+08	2.29E+10
		Live	1.14E+08	1.22E+08		1.18E+08	1.95E+10
		Dead	1.94E+07	2.26E+07		2.10E+07	3.47E+09
		% Viability	85.40%	84.40%		84.90%	

Figure 106

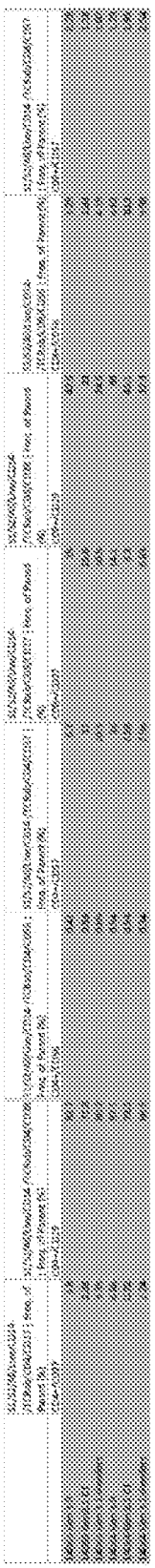


Figure 107

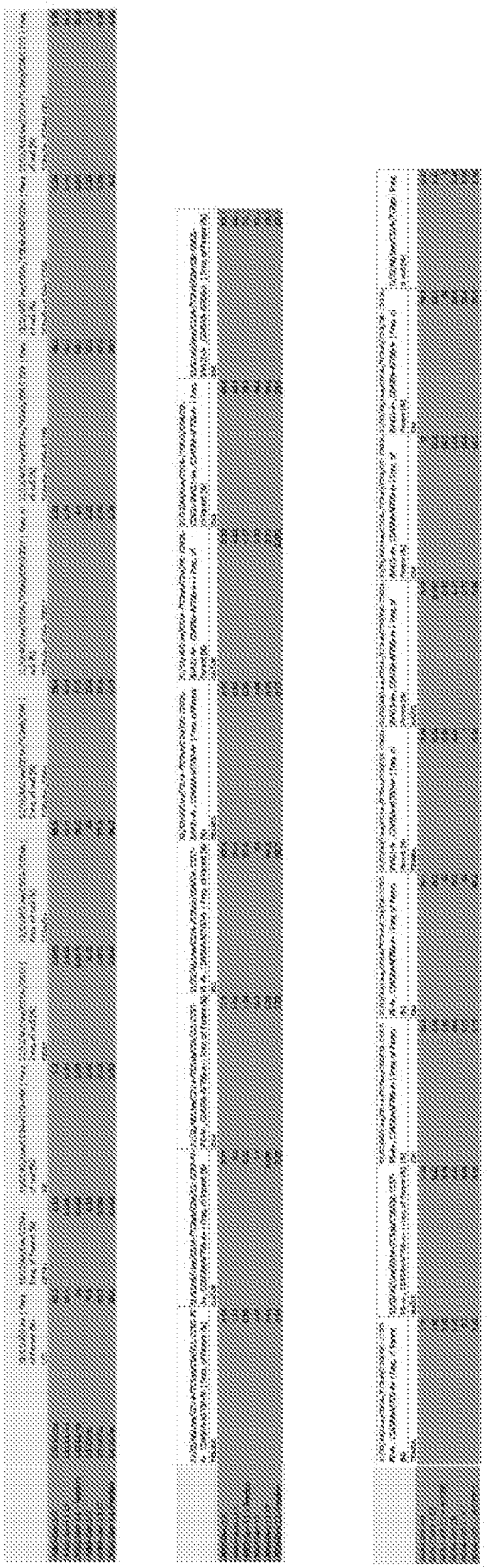


Figure 1108

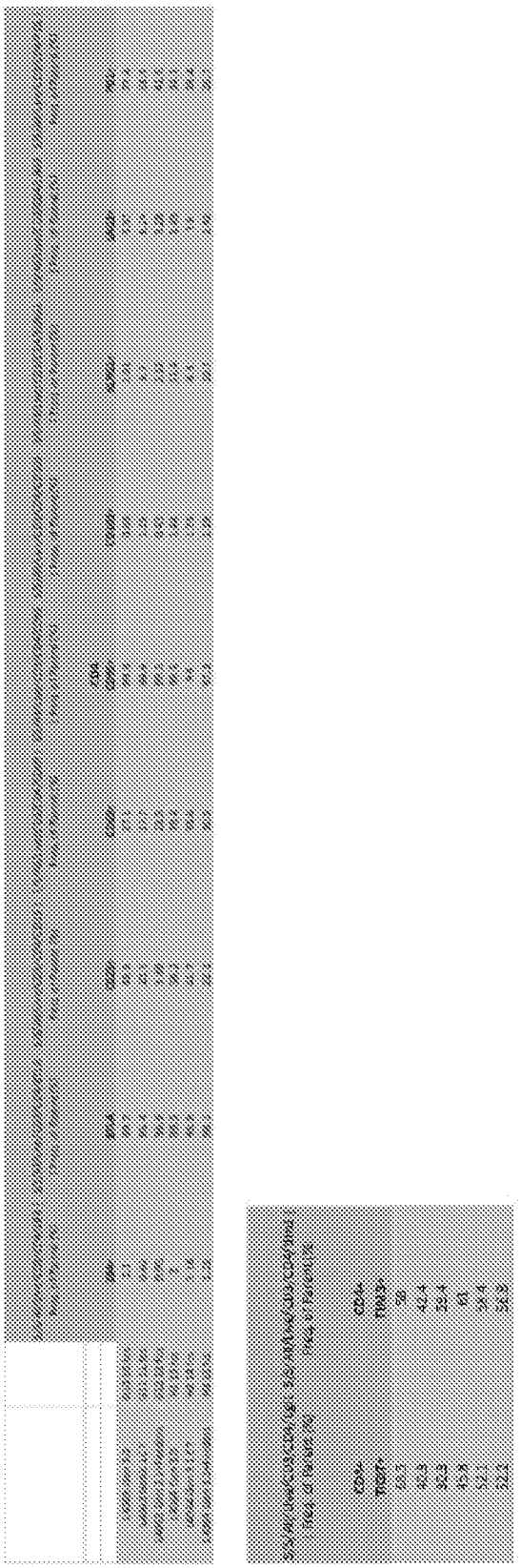


Figure 109

Figure 110

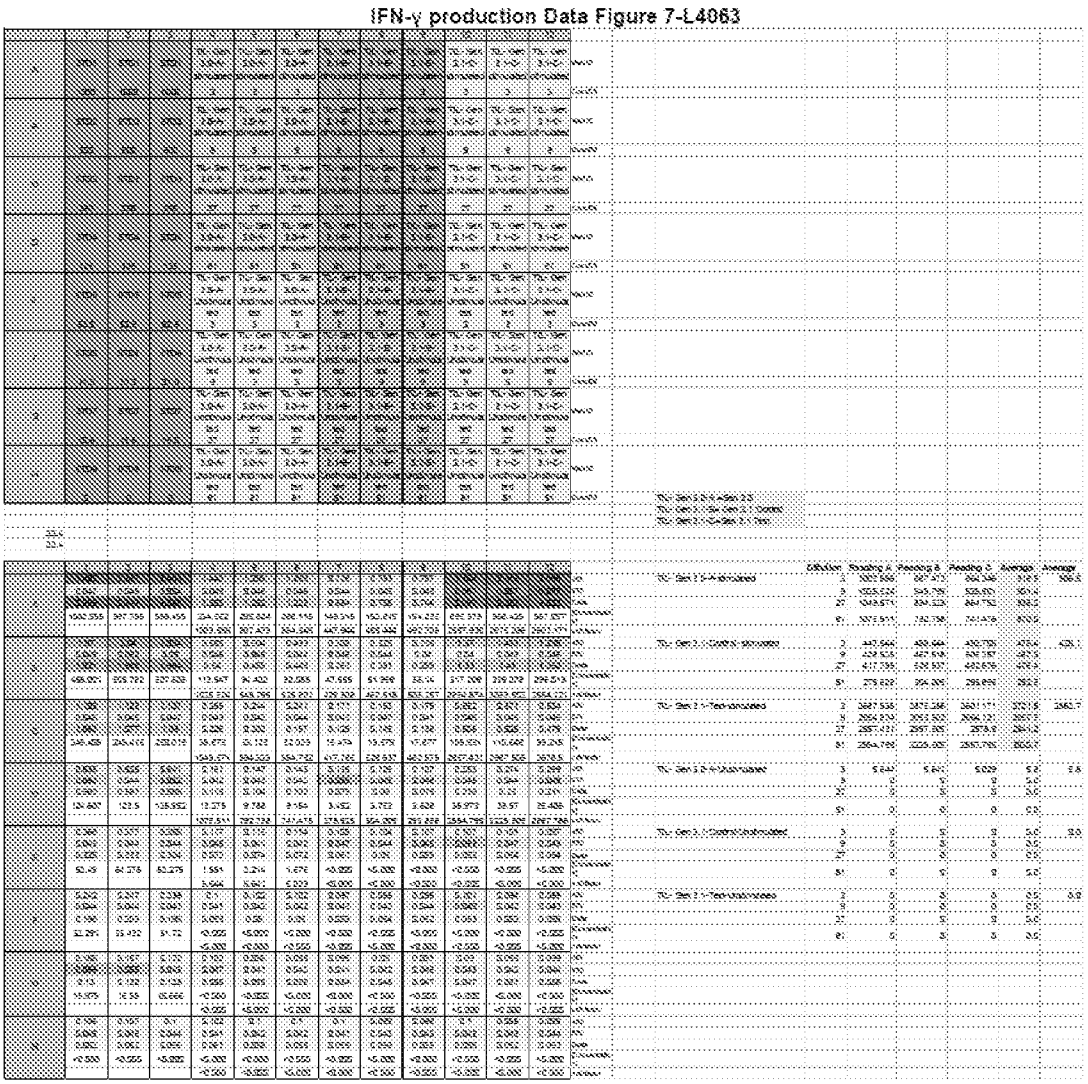


Figure 111

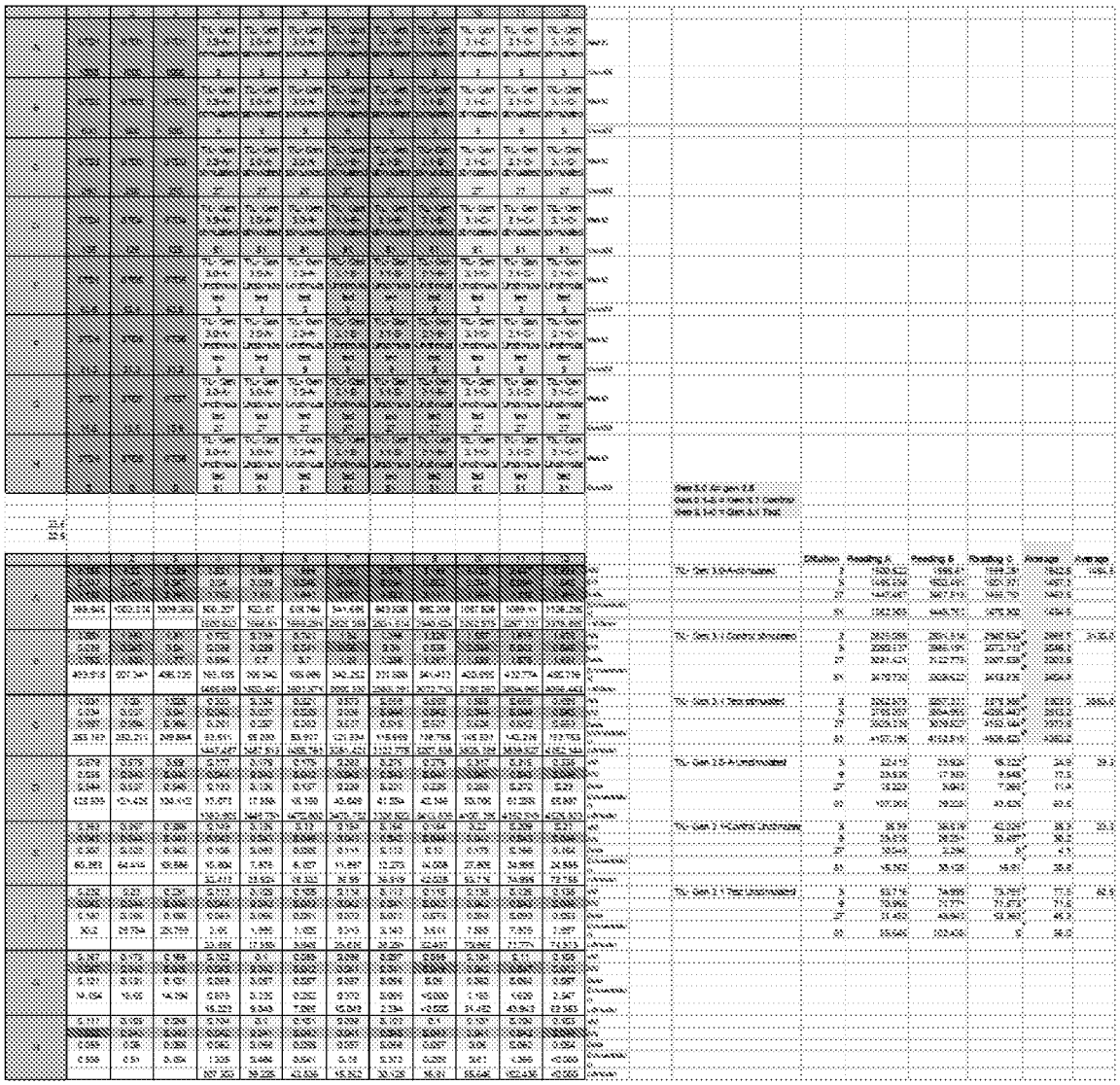


Figure 112

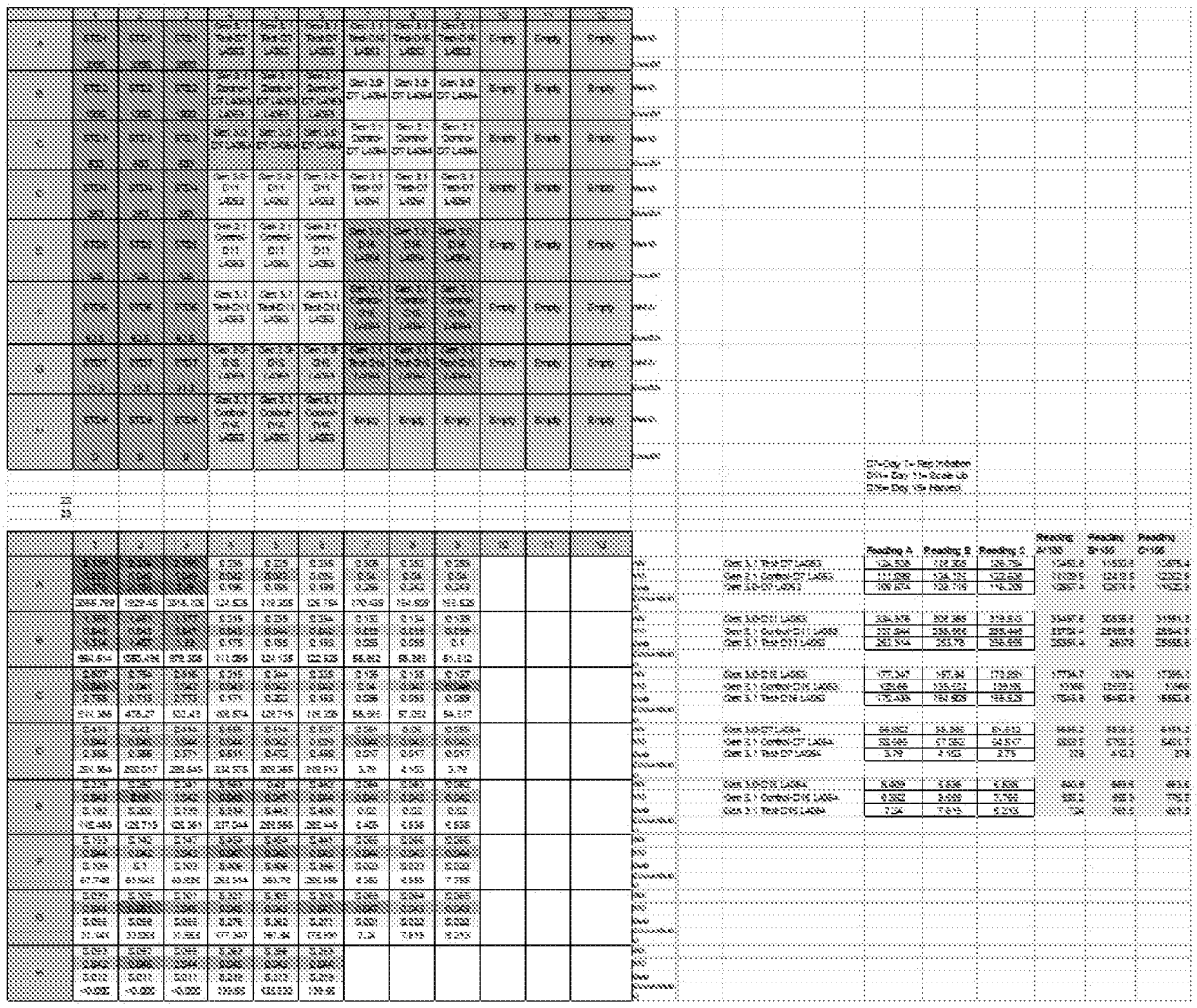


Figure 113

Sample number #	Description Gen 3.1 Optimization	Glucose (g/L)	Lactate (g/L)	Ammonia (mmol/L)	Glutamine (mmol/L)	Glutamax (mmol/L)	Glutamax-Glutamine (mmol/L)
13	Gen 3.0-Day 16 First Round #L4063	1.52	1.07	2.06	1.91	2.11	0.20
14	Gen 3.1 Control-Day 16 First Round #L4063	0.90	1.74	2.50	1.34	1.49	0.15
15	Gen 3.1 Test-Day 16 First Round #L4063	0.96	1.64	2.49	1.33	1.47	0.14
16	Gen 3.0-Day 11 First Round #L4063	1.42	0.70	2.83	1.70	1.86	0.16
17	Gen 3.1 Control-Day 11 First Round #L4063	0.90	1.08	2.24	1.07	1.19	0.11
18	Gen 3.1 Test-Day 11 First Round #L4063	0.84	1.21	2.25	1.11	1.23	0.12
19	Gen 3.0-Day 7 First Round #L4063	1.76	0.20	1.88	2.04	2.23	0.19
20	Gen 3.1 Control-Day 7 First Round #L4063	1.73	0.22	1.86	2.00	2.19	0.19
21	Gen 3.1 Test-Day 7 First Round #L4063	1.41	0.50	1.96	1.98	2.15	0.17
22	Gen 3.0-Day 16 Second Round #L4064	2.76	1.52	1.00	0.68	0.77	0.09
23	Gen 3.1 Control-Day 16 Second Round #L4064	2.14	1.95	0.99	0.64	0.73	0.08
24	Gen 3.1 Test-Day 16 Second Round #L4064	2.20	1.89	0.94	0.64	0.71	0.07
25	Gen 3.0-Day 7 Second Round #L4064	4.26	0.08	0.71	1.29	1.43	0.14
26	Gen 3.1 Control-Day 7 Second Round #L4064	4.14	0.22	0.78	1.17	1.29	0.13
27	Gen 3.1 Test-Day 7 Second Round #L4064	3.82	0.49	0.89	1.09	1.20	0.11

Figure 114

Order	Name		Geometric Mean Probe-fitc-A	Geometric Mean Probe-fitc-A	Average 1301	Average T	Length relative to 1301	
A1	Gen 3.0-L4063	A1 10.1.fcs	86	42.5				
A2		A2 10.2.fcs	85.4	42.3	85.7	42.4		
A3		A3 10.3.fcs	4585	207				
A4		A4 10.4.fcs	4389	216	4487	211.5	7.684093	
A5	Gen 3.1 Control-L4063	A5 11.1.fcs	82.7	33.3				
A6		A6 11.2.fcs	86	32.9	84.35	33.1		
A7		A7 11.3.fcs	4696	180				
A8		A8 11.4.fcs	4641	175	4668.5	177.5	6.299968	
B1	Gen 3.1 Test-L4063	B1 12.1.fcs	95.1	28.8				
B2		B2 12.2.fcs	84.7	26.7	89.9	27.75		
B3		B3 12.3.fcs	4764	169				
B4		B4 12.4.fcs	4800	174	4782	171.5	6.12732	
B5	Gen 3.0 -L4064	B5 13.1.fcs	101	40.3				
B6		B6 13.2.fcs	89.6	34.1	95.3	37.2		
B7		B7 13.3.fcs	4300	174				
B8		B8 13.4.fcs	4367	172	4333.5	173	6.408381	
C1	Gen 3.1 Control-L4064	C1 14.1.fcs	92.1	31.1				
C2		C2 14.2.fcs	88.6	29.6	90.35	30.35		
C3		C3 14.3.fcs	4111	169				
C4		C4 14.4.fcs	4443	172	4277	170.5	6.69509	
C5	Gen 3.1 Test-L4064	C5 15.1.fcs	89.7	25.7				
C6		C6 15.2.fcs	85.7	24.7	87.7	25.2		
C7		C7 15.3.fcs	4342	172				
C8		C8 15.4.fcs	4560	166	4451	169	6.591341	

Figure 115

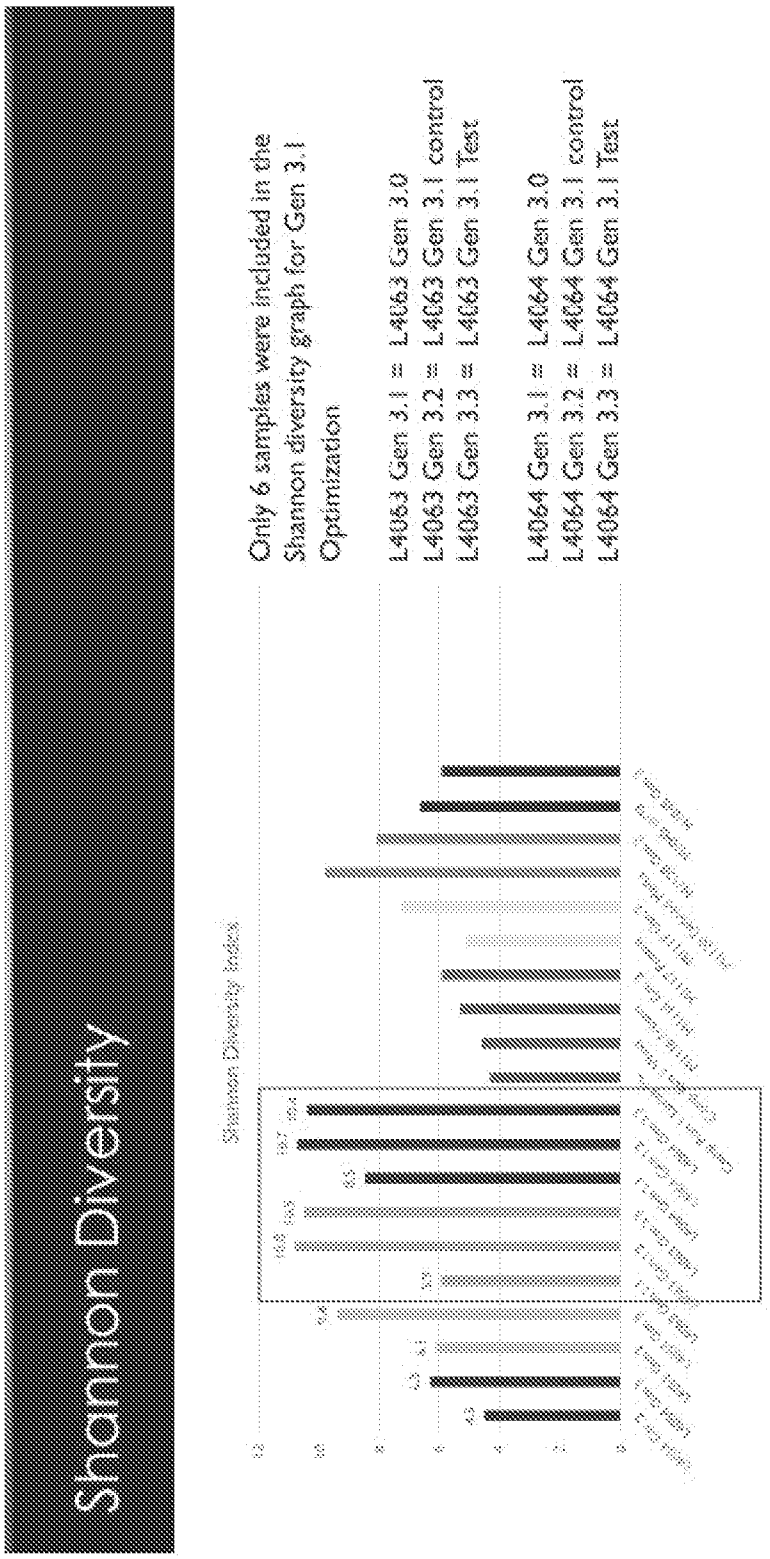
Shared Frequencies

Subject	Original Sample	uCDR3 count	Compared Sample	uCDR3 count	shared uCDR3 count	% shared of B
L4063	L63GA	14026	L63GB	18142	3240	23.13493479
L4063	L63GA	14026	L63GC	17181	1959	11.12816491
L4064	L64GA	15207	L64GB	21460	5486	36.31429139
L4064	L64GA	15207	L64GC	21509	5541	36.67836103
Code:	L63GA = L4063 Gen 3.0					
	L63GB = L4063 Gen 3.1 control					
	L63GC = L4063 Gen 3.1 Test					
	L64GA = L4064 Gen 3.0					
	L64GB = L4064 Gen 3.1 control					
	L64GC = L4064 Gen 3.1 Test					

Top 80%

subject	sample1	uCDR3 count	sample2	uCDR3 counts	shared uCDR3 count	%shared of col B
L4063	L63GA	1104	L63GB	2437	1000	90.57971
L4063	L63GA	1104	L63GC	2331	975	88.31522
L4064	L64GA	2478	L64GB	3246	2186	88.2163
L4064	L64GA	2478	L64GC	3263	2163	87.28814
Code:	L63GA = L4063 Gen 3.0					
	L63GB = L4063 Gen 3.1 control					
	L63GC = L4063 Gen 3.1 Test					
	L64GA = L4064 Gen 3.0					
	L64GB = L4064 Gen 3.1 control					
	L64GC = L4064 Gen 3.1 Test					

Figure 116


Shannon Diversity
Shannon Diversity Index
Only 6 samples were included in the Shannon diversity graph for Gen 3.1 Optimization
L4063 Gen 3.1 = L4063 Gen 3.0
L4063 Gen 3.2 = L4063 Gen 3.1 control
L4063 Gen 3.3 = L4063 Gen 3.1 Test
L4064 Gen 3.1 = L4064 Gen 3.0
L4064 Gen 3.2 = L4064 Gen 3.1 control
L4064 Gen 3.3 = L4064 Gen 3.1 Test