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(54) **Titre : COMPOSITIONS ET PROCEDES POUR L'EXPANSION DE LYMPHOCYTES T ET DE LYMPHOCYTES INFILTRANT LES TUMEURS**

(54) **Title: COMPOSITIONS AND METHODS FOR EXPANSION OF T CELLS AND TUMOR INFILTRATING LYMPHOCYTES**

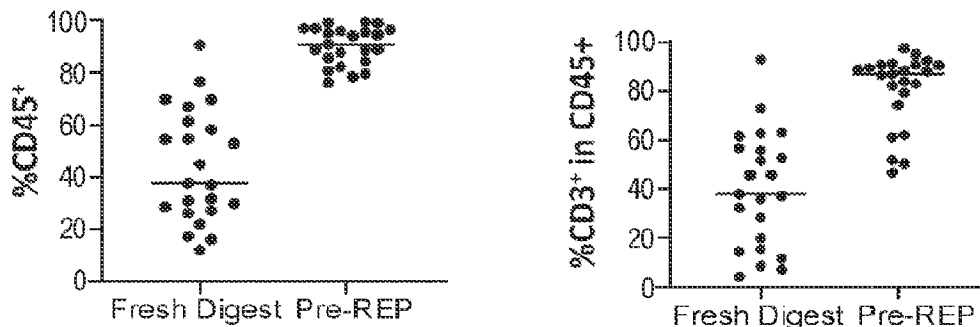


FIG. 1

(57) **Abrégé/Abstract:**

The present disclosure provides compositions and methods for expanding T cells or tumor infiltrating lymphocytes (TILs) in vitro. K562 feeder cells engineered to express a costimulatory molecule (e.g., 41BB ligand (41BBL)) and either interleukin 21 (IL21) or interleukin 7 (IL7) can be used in a rapid expansion protocol (REP) step to expand the T cells or TILs. Thus, provided herein is a culture comprising T-cells or TILs and modified K562 feeder cells. The T cells can be modified to express a chimeric antigen receptor (CAR) or a T cell receptor (TCR) or the TILs can be modified to express membrane-bound IL15 (mbIL15). The T cells or TILs can be expanded in vitro using a REP without the use of exogenous interleukin 2 (IL2), and the expanded cells can be used in adoptive cell therapy for treatment of cancer without concomitant use of an exogenous cytokine such as IL2.

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Abstract:

The present disclosure provides compositions and methods for expanding T cells or tumor infiltrating lymphocytes (TILs) in vitro. K562 feeder cells engineered to express a costimulatory molecule (e.g., 41BB ligand (41BBL)) and either interleukin 21 (IL21) or interleukin 7 (IL7) can be used in a rapid expansion protocol (REP) step to expand the T cells or TILs. Thus, provided herein is a culture comprising T-cells or TILs and modified K562 feeder cells. The T cells can be modified to express a chimeric antigen receptor (CAR) or a T cell receptor (TCR) or the TILs can be modified to express membrane-bound IL15 (mbIL15). The T cells or TILs can be expanded in vitro using a REP without the use of exogenous interleukin 2 (IL2), and the expanded cells can be used in adoptive cell therapy for treatment of cancer without concomitant use of an exogenous cytokine such as IL2.

COMPOSITIONS AND METHODS FOR EXPANSION OF T CELLS AND TUMOR INFILTRATING LYMPHOCYTES

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to U.S. Provisional Application No. 63/139,305, filed January 19, 2021; U.S. Provisional Application No. 63/153,367, filed February 24, 2021; U.S. Provisional Application No. 63/226,114, filed July 27, 2021; U.S. Provisional Application No. 63/244,166, filed September 14, 2021, which are hereby incorporated by reference in their entireties for all purposes.

BACKGROUND

[002] Adoptive cell therapy (ACT) using T cells or tumor infiltrating lymphocytes (TILs) is emerging as a clinical tool for the treatment of subjects that are immunocompromised or subjects with cancer. T cells prepared from peripheral blood mononuclear cells (PBMC) or TILs prepared from a tumor have been used in ACT with varying degrees of success. Typically, *in vitro* expansion of the cell population is required prior to infusion into a subject. Conventional expansion includes a two-step process, the first step referred to as the pre-rapid expansion protocol (pre-REP) step and the second step referred to as the rapid expansion protocol (REP) step. The pre-REP step requires that the isolated cells be cultured in the presence of interleukin 2 (IL2) but in the absence of feeder cells, whereas the REP step typically requires feeder cells (e.g., peripheral blood mononuclear cells (PBMCs)), high doses of IL2 and, optionally, anti-CD3 antibody (OKT3). Although conventional expansion works for most T cells or TILs, genetically modified cells may require a modified expansion process in order to selectively expand the population of modified T cells or TILs. Furthermore, expanded T cells or TILs when administered to a patient typically receive concurrent administration of IL2 to activate and expand the T cell or TIL population *in vivo*. But, IL2 shows dose-dependent toxicity, which can manifest in multiple organ systems, most significantly the heart, lungs, kidneys, and central nervous system. The most common manifestation of IL-2 toxicity is capillary leak syndrome, resulting in a hypovolemic state and fluid accumulation in the extravascular space. A significant number of patients will not tolerate the adjunct IL2 treatment and therefore must be excluded from TIL treatment. Improvements in the field are needed to make ACT a safe and more effective therapeutic tool.

SUMMARY

[003] This disclosure relates to compositions and methods for expanding modified T cells or TILs *in vitro*. K562 feeder cells engineered to express a costimulatory molecule (e.g., 41BB ligand (41BBL)) and either interleukin 21 (IL21) or interleukin 7 (IL7) can be used in the REP stage to expand modified T cells or TILs, particularly T cells or TILs that do not expand well under conventional REP conditions. Thus, provided herein is a culture comprising modified T-cells or TILs and modified K562 feeder cells. The K562 feeder cells are optionally replication incompetent and comprise a first exogenous nucleic acid sequence encoding a costimulatory molecule chosen from the tumor necrosis factor superfamily (e.g., 41BBL) and a second exogenous nucleic acid sequence encoding IL21 or IL7. The T-cells or TILs are modified T cells or TILs. By way of example the culture can comprise T cells or TILs that are modified to express a cytokine (such as IL15 or a membrane-bound IL15 (mbIL15)), a chimeric antigen receptor (CAR), and/or a T cell receptor (TCR). Optionally, one or more of the CAR, TCR, or mbIL15 are optionally operably linked to a drug responsive domain (DRD), and the K562 feeder cells optionally express 41BBL and IL21 (e.g., membrane-bound IL21).

[004] DRDs are polypeptides that can regulate the abundance and/or activity of a payload, such as mbIL15, upon binding with a ligand. Multiple DRDs, for example, in series, can regulate a single payload. The one or more DRDs are operably linked to the mbIL15, CAR, or TCR payload such that interaction of the DRD with an effective amount of ligand under appropriate conditions results in modifying the biological activity or amount of the payload.

[005] Also provided is a method of expanding modified T cells or TILs by culturing the modified T cells or TILs in the presence of a population of modified K562 feeder cells, wherein the modified K562 feeder cells comprise a first exogenous nucleic acid sequence encoding a costimulatory molecule chosen from the tumor necrosis factor superfamily (e.g., 41BBL) and a second exogenous nucleic acid sequence encoding IL21 or IL7. This method allows the REP stage to proceed in the absence of exogenous IL2. The T cells or TILs expanded with this method are modified (engineered). By way of example, the method can be used to expand TILs that are modified to express membrane-bound IL15, which can be operably linked to a DRD, or to expand T cells that are modified to express a cytokine, a CAR, and/or a TCR, any of which can be operably linked to a DRD.

[006] In the method of expanding modified T cells or TILs, the modified K562 feeder cells can be replication incompetent and can be engineered to express IL21 (e.g., secreted IL21 or

a membrane-bound IL21). Furthermore, the K562 feeder cells can be engineered to express 41BBL.

[007] Also provided herein is a method of expanding TILs engineered to express mbIL15 by culturing the TILs in the presence of modified K562 feeder cells. The TILs engineered to express mbIL15 expand in the absence of exogenous cytokines (e.g., exogenous interleukins like IL2, IL7, IL15, or variants thereof. The modified K562 feeder cells can be replication incompetent, modified to express a costimulatory molecule chosen from the tumor necrosis factor superfamily (e.g., 41BBL), and/or modified to express IL21 or IL7. Optionally, in the method of expanding TILs, the K562 feeder cells are modified to express mbIL21.

[008] An expanded population of T cells or TILs is provided. The population of modified T cells or TILs are expanded in the presence of K562 feeder cells, which are replication incompetent, modified to express a costimulatory molecule chosen from the tumor necrosis factor superfamily (e.g., 41BBL), and/or modified to express IL21 (e.g., mbIL21) or IL7 (e.g., mbIL7). Modified T cells and TILs expanded by the methods described herein have certain advantageous properties. The absence of IL2 in the REP step has the advantage of producing TILs or T cells that are less differentiated or exhausted than unengineered TILs or T cells expanded in the presence of IL2. additionally, TILs produced according to the methods described herein can be administered to patients in need without the need for concomitant IL2 therapy, which is toxic to many patients and which also exhausts the T cells or TILs. For example, the expanded modified T cells or TILs survive (persist) more than unengineered cells. The population of expanded T cells or TILs has a greater proportion of CD8⁺ cells and a lower proportion of CD4⁺ cells as compared to the proportion of CD8⁺ cells and CD4⁺ cells in a control population of unexpanded T cells or TILs. Thus, the population of expanded T cells or TILs has a CD4:CD8 ratio lower than the CD4:CD8 ratio of a control population of unexpanded TILs or TILs or T-cells expanded on allogeneic PBMCs. Additionally, the population of expanded mbIL15 T cells or TILs has a lesser proportion of T_{reg} cells as compared to the proportion of T_{reg} cells in pre-REP T cells or TILs. The population of expanded T cells or TILs also has a lesser proportion of PD1⁺ cells as compared to the proportion of PD1⁺ cells in a control population of unexpanded T cells or TILs or in a control population of TILs or T-cells expanded on PBMCs. The population of expanded T cells or TILs as described herein also has a greater proportion of polyfunctional cells producing for tumor necrosis factor α (TNF α) and interferon γ (IFN γ) as compared to the proportion of T cells and TILs producing TNF α and IFN γ in a control population of

unexpanded TILs. Also, modified T cells (e.g., T cells modified to express a cytokine, CAR, or TCR) or modified TILs (e.g., TILs modified to express a mbIL15) expand in the presence K562 feeder cells, 41BB ligand (41BBL), and interleukin 21 (IL21, secreted or membrane bound to the K562 feeder cells) more than unmodified TILs. The preferential expansion of modified T cells or TILs occurs in the absence of exogenous cytokines, like IL2.

[009] Provided herein is a method of treating cancer in a subject by administering to the subject an expanded population of modified T cells or modified TILs expanded according to the methods described herein. Optionally, the T cells or TILs are expanded in the presence of K562 feeder cells, which are replication incompetent, modified to express a costimulatory molecule chosen from the tumor necrosis factor superfamily (e.g., 41BBL), and/or modified to express IL21 (e.g., mbIL21) or IL7 (e.g., mbIL7). The expansion step is optionally in the absence of IL2. Additionally, the expanded cells can be administered to the subject without administration of exogenous IL2. Systemic administration of IL2 to cancer patients concomitant with or following immunotherapy often causes toxicity in patients who are already medically fragile. Many patients suffer severe, life-threatening side effects after IL2 administration, including hypotension and shock due to capillary leakage syndrome. TIL therapy with low doses of concomitant IL2 has been attempted, but the immunotherapy was less effective than when IL2 was administered at higher doses. Thus the modified T cell or modified TIL expanded according to the methods described herein can be used in a treatment regimen that proves less toxic to a subject with cancer than current treatment regimens that require the use of exogenous IL2. T cells or TILs expanded according to the methods taught herein and administered to a subject can be further engineered such that the T cells are engineered to express a cytokine (e.g., IL15), CAR, or TCR operably linked to one or more DRDs or such that the TILs are engineered to express mbIL15 is operably linked to one or more DRD.

[0010] The identified embodiments are exemplary only and are therefore non-limiting. The details of one or more non-limiting embodiments of the invention are set forth in the accompanying drawing and the description below. Other embodiments of the invention should be apparent to those of ordinary skill in the art after consideration of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] **FIG. 1** shows frequency of CD45⁺ cells (left) and CD3⁺ T cells within CD45⁺ cells (right) in fresh tumor digest and after 3 weeks pre-REP TIL culture.

[0012] **FIG. 2** shows transduction efficiency of IL15-293 construct in two melanoma TIL donors measured by flow cytometry on day 5 post-transduction.

[0013] **FIG. 3A-B** show antigen and IL2-independent expansion and survival of TILs expressing mbIL15. **FIG. 3A** shows TIL donor 006 cells (TIL 006) transduced with constitutive mbIL15 or GFP and expanded in REP for 12 days with or without 6000 IU/mL IL2. **FIG. 3B** shows TIL 006 transduced with constitutive mbIL15 (expanded in REP without IL2) or GFP (expanded in REP with 6000 IU/mL IL2) and enumerated in a 14-day antigen-independent survival assay, with and without 6000 IU/mL IL2.

[0014] **FIG. 4** shows antigen-independent TIL expansion after a rapid expansion protocol (REP). After REP, unengineered and mbIL15 engineered TILs (constitutive or regulated mbIL15) were plated with or without exogenous IL2 or acetazolamide (ACZ), and new wells were harvested every 3 days to assess cell enumeration and phenotype.

[0015] **FIG. 5** shows TIL expansion in an antigen-dependent setting. After a rapid expansion protocol (REP), unengineered and mbIL15 engineered TILs were plated with HLA-matched mitomycin C-treated melanoma cells in a TIL:tumor co-culture assay with and without exogenous IL2, acetazolamide, or vehicle (DMSO) and wells were harvested every 3 days to assess cell enumeration and phenotype.

[0016] **FIG. 6A-B** show tumor reactivity of TILs after a rapid expansion protocol (REP). **FIG. 6A** shows TIL 006 and TIL 005, both transduced with regulated mbIL15 and unengineered controls and co-cultured for 24-hours with HLA- matched mitomycin-C treated melanoma cells. IFN γ in supernatants was measured by MSD assay. **FIG. 6B** shows cytotoxicity of TILs in co-culture as measured by loss of luminescence by luciferase-tagged HLA-matched melanoma line.

[0017] **FIG. 7A-B** show TIL expansion and transduction efficiency prior to infusion into animals for an *in vivo* adoptive cell therapy experiment. **FIG. 7A** shows cell expansion for TIL donor 006, used for *in vivo* adoptive cell transfer (ACT), of unengineered and mbIL15 engineered TILs. **FIG. 7B** shows transduction efficiency after a rapid expansion protocol (REP); unengineered and mbIL15 engineered TILs were assessed for expression of IL15 and IL15RaFc as a measure of transduction efficiency.

[0018] **FIG. 8A-C** show analyses of TIL enumeration and IL15 expression for *in vivo* adoptive cell therapy experiment. **FIG. 8A** shows enumeration of adoptively transferred unengineered and mbIL15 engineered TILs by flow cytometry from peripheral blood samples. TILs were identified as live humanCD3+murineCD45- cells in submandibular vein

blood samples. FIG. 8B and FIG. 8C show TIL enumeration (hCD3+mCD45-) and IL15 expression (IL15+IL15RaFc+) of splenic and bone marrow samples isolated 14 days or 53 days after ACT.

[0019] FIG. 9 shows acetazolamide (ACZ) regulation of IL15 expression and signaling in cryopreserved regulated mbIL15 TILs occurs in a dose-dependent fashion. Regulated mbIL15 TILs from four patients (Patients 1-4) were thawed and rested in ACZ-free media for 24 hours, then regulated in 0.1, 1, 2.5, 5, 10, 25, 100 μ M of ACZ for 18 hours. Regulated mbIL15 TILs were then collected and analyzed for IL15 expression and signaling using a phospho-flow cytometry-based assay. FIG. 9A shows the frequency of IL15+ TILs as a percentage of CD3+ cells. FIGs. 9B-9E show the results for each patient: here, cells were further gated on IL15+, and then geometric mean fluorescent intensity for each pSTAT5 (open square) and pS6 (closed circle) was calculated. Values shown are set relative to vehicle control. N=4 human donors.

[0020] FIG. 10 shows the mean fluorescence intensity (MFI) for pSTAT5 and pS6 in patients 1-4. FIG. 10A shows the MFI for pSTAT5. FIG. 10B shows the MFI for pS6.

[0021] FIG. 11 shows constitutive mbIL15 expression and ACZ regulation of regulated mbIL15 TILs engage the IL15 signaling pathway. Here, unengineered TILs and regulated mbIL15 TILs from Patients 1-3, were thawed and rested in ACZ-free media for 24 hours, then regulated with IL2 or ACZ for 18 hours. Cells were then collected and analyzed for IL15 expression and signaling using a phospho-flow cytometry-based assay. Unengineered TILs and regulated mbIL15 TILs +vehicle were gated on Live cells followed by singlets, followed by CD3+. Constitutive IL15 TILs and regulated mbIL15 TILs +ACZ conditions were further gated on IL15+ staining. Geometric mean fluorescent intensity for each pSTAT5 and pS6 was calculated. N=3 human donors.

[0022] FIG. 12 shows regulated mbIL5-modified TILs without exogenous cytokines demonstrate greater polyfunctionality than unengineered TILs +IL2. Unengineered TILs and regulated mbIL15 TILs were thawed and rested in ACZ-free media for 24 hours; next, the unengineered TILs were treated with the following concentrations of IL2: 20, 200, 1000 and 6000 IU/mL, or vehicle; and regulated mbIL15 TILs were treated with the following concentrations of ACZ: 0.1, 1, 5, 10, 25, 100 μ M ACZ, or vehicle. Treatments were for 18 hours. Cells were stimulated with PMA and ionomycin for 6 hours in the presence of brefeldin A and monensin. Unstimulated TILs were used as controls (data not shown). After stimulation, cells were analyzed for expression of IL15 and intracellular TNF α and IFN γ

using a flow cytometry-based assay. TILs were gated on Live cells, followed by singlets, followed by CD3+, and regulated mbIL15 TILs were additionally gated on IL15+. FIG. 12A shows TNF α and IFN γ double positive populations for unengineered TILs with IL2, and regulated mbIL15 TILs with ACZ. FIG. 12B shows IL15 expression in regulated mbIL15 TILs cultures. FIG. 12C shows a comparison of select IL2 (200 IU/mL) and ACZ (25 μ M) doses.

[0023] FIG. 13 shows the results of a patient-derived xenograft (PDX) efficiency model. At the end of the end of the rapid expansion protocol (REP), unengineered TILs and regulated mbIL15 TILs (+/- acetazolamide (ACZ)) were adoptively transferred into mice bearing a human melanoma PDX. Mean tumor volumes were evaluated (+/- SEM). FIG. 13A shows mean tumor volume for a given treatment at days post adoptive cell transfer (ACT). FIG. 13B shows tumor volume at days post ACT for no TILs (top left); unengineered TILs + IL2 (top right); regulated mbIL15 TILs + vehicle (bottom left); and regulated mbIL15 TILs +ACZ (bottom right). Here, regulated mbIL15 TILs + ACZ significantly superior anti-tumor efficacy compared to unengineered TIL + IL2 (*p<0.05; Mann U Whitney).

[0024] FIG. 14 shows the results of a SK-MEL-1 xenograft cancer model. At the end of the end of the rapid expansion protocol (REP), unengineered TILs and regulated mbIL15 TILs (+/- acetazolamide (ACZ)) were adoptively transferred into mice bearing SK-MEL-1 tumors. Mean tumor volumes were evaluated (+/- SEM). FIG. 14A shows mean tumor volume for a given treatment at days post adoptive cell transfer (ACT). FIG. 14B shows tumor volume at days post ACT for no TILs (top left); unengineered TILs + IL2 (top right); regulated mbIL15 TILs + vehicle (bottom left); and regulated mbIL15 TILs +ACZ (bottom right). Here, regulated mbIL15 TILs + ACZ show significantly superior anti-tumor efficacy compared to unengineered TIL + IL2 (*p<0.05; Mann U Whitney).

[0025] FIG. 15 shows regulated mbIL15 TILs achieve enhanced MHC-I-dependent cytotoxicity against melanoma *in vitro*. Here, unengineered TILs and regulated mbIL15 TILs were cryopreserved at the end of the rapid expansion protocol (REP). Cryopreserved TILs were thawed and rested in cytokine-free conditions overnight, and then co-cultured with Cell Trace Violet-labeled melanoma cells (SK-MEL-1) at a 1:1 and 5:1 effector-to-target (TIL:melanoma) ratios. To control for MHC-I dependent cytotoxicity, melanoma cells were pre-treated with 80 μ g/mL HLA ABC MHC blocking antibody for 2 hours prior to the assay. After 3 hours of co-culture, the SK-MEL-1 cells were evaluated for expression of intracellular cleaved-caspase 3 (a marker for irreversible commitment to cell death) by flow

cytometry. Quantified cleaved caspase 3 was normalized to that of target cells alone (spontaneous or background release). Bar graphs show expression of cleaved capsase-3 on target tumor cells when co-cultured with TILs from 6 individual patients.

[0026] FIG. 16 is a graph showing that maximal TIL expansion in REP occurs when mbIL15 TILs (constitutive) are generated with K562 feeder cells with both IL21 and 41BBL-mediated co-stimulation.

[0027] FIG. 17 is a graph showing that maximal TIL expansion in REP occurs when unengineered TILs are generated with pooled PBMC feeders or K562 feeder cells expressing membrane-bound IL21 and 41BBL.

[0028] FIG. 18 shows that maximal expansion of IL15+ TILs in REP occurs when TILs with mbIL15 (constitutive) are generated with K562 feeder cells and receiving both IL21 and 41BBL-mediated co-stimulation. Results on feeder cells at days 8, 11, 15, and 18 are shown from left to right: PBMC feeders, K562-parental feeders, K562 + 41BBL, K562 + 41BBL feeders with recombinant human IL21, K562 + mbIL21 feeders, K562 + 41BBL+mbIL21 feeders.

[0029] FIG. 19 is a graph showing that IL15 expression is enriched through the REP process in mbIL15 TILs (constitutive) generated with K562 feeder cells and receiving both IL21 and 41BBL-mediated co-stimulation.

[0030] FIG. 20 is a graph showing expanded TILs with mbIL15 generated with K562 feeder cells with both IL21 and 41BBL-mediated co-stimulation have a decreased CD4:CD8 ratio throughout REP. Thus, TILs with mbIL15 expanded in the presence of K562 feeder cells with both IL21 and 41BBL stimulation are enriched for CD8+ cytotoxic effector cells, in contrast to expanded TILs with mbIL15 generated with pooled PBMC feeders, unmodified K562 feeders, or K562 feeders expressing 41BBL in the absence of IL21. CD4:CD8 ratios are shown at days 8, 11, 15, and 18 from left to right: PBMC feeders, K562-parental feeders, K562 + 41BBL, K562 + 41BBL feeders with recombinant human IL21, K562 + mbIL21 feeders, K562 + 41BBL+mbIL21 feeders.

[0031] FIG. 21 is a graph showing a higher percentage of TNF α +interferon γ + cells in expanded mbIL15 TILs generated with K562 feeder cells expressing both mbIL21 and 41BBL, as compared to mbIL15 TILs generated with PBMC feeder cells or unmodified K562 feeder cells. The higher percentage of TNF α +interferon γ + TILs is indicative of enhanced polyfunctionality in expanded mbIL15 TILs generated with K562 feeder cells expressing both mbIL21 and 41BBL.

[0032] FIG. 22 is a graph showing the results of a 10-day survival assay for mbIL15 TILs generated with PBMC feeder cells, unmodified K562 feeder cells, K562 feeder cells expressing only mb41BBL, K562 feeder cells expressing only mbIL21, K562 feeder cells expressing both 41BBL and mbIL21, and K562 feeder cells expressing 41BBL in the presence of recombinant human IL21. Expanded mbIL15TILs generated with K562 feeder cells and receiving both IL21 and 41BBL-mediated co-stimulation demonstrated improved post-REP antigen-independent survival as compared to mbIL15 TILs generated with PBMC feeder cells or K562 feeder cells that are unmodified or modified to express mbIL21 or 41BBL independently.

[0033] FIG. 23 shows the relative proportion of TCRV β subfamilies in unengineered TILs and mbIL15 TILs expanded under with PBMC feeders, K562 feeders, K562+mbIL21 feeders, K562+41BBL feeders, K562+41BBL+mbIL21 feeders, or K562+41BBL+rhIL21 feeders. Expanded mbIL15 TILs and unengineered TILs maintain diverse subfamily distribution regardless of feeder cells or conditions.

[0034] FIG. 24 shows the expression of PD1 on the surface of mbIL15 TIL, as gated on live CD3⁺ cells from left to right in unexpanded TIL, and expanded TIL generated with PBMC feeders, K562-parental feeders, K562 + 41BBL feeders, K562 + 41BBL feeders with recombinant human IL21, K562 + mbIL21 feeders, and K562+41BBL+mbIL21 feeders. PD1 expression is highest in unexpanded mbIL15 TIL, and expansion of mbIL15 TILs with both 41BBL and IL21-mediated signaling produces TILs with near baseline expression of PD1.

[0035] FIG. 25 shows phenotyping comparing pre-REP TILs (as described in Example 1) to engineered mbIL15 TILs (as described in Example 3). Pre-REP and post-REP TILs were phenotyped by flow cytometry using antibodies for CD3, CD4, CD8, and PD1 as described in Example 13. As shown in FIG. 25A, the frequency of CD8⁺ T cells is higher and the frequency of CD4⁺ T cells is lower for post-REP mbIL15 TILs as compared with corresponding pre-REP TILs from the same TIL donors. In FIG. 25B, the post-REP mbIL15 TILs express lower levels of PD1 than corresponding pre-REP TILs from the same TIL donors. FIG 25C shows the percentages of a regulatory T cell population in mbIL15 TIL, identified as CD3⁺ T cells that are gated as CD4⁺ and further classified as CD25 and FoxP3 double positive cells. mbIL15 TILs have a reduced proportion of of regulatory T cells as compared to preREP TILs prior to the engineering step.

[0036] **FIG. 26** shows the expression of conserved melanoma-associated antigens MART-1 and gp100 on the A375 melanoma cell line and on patient-derived xenograft (PDX) cells (PDX163A, described in Example 11), as determined by flow cytometry.

[0037] **FIG. 27** shows the percentage of MART-1-tetramer positive TILs and gp100-tetramer positive TILs in mbIL15 TIL derived from four distinct TIL donors that are HLA-matched to PDX 163A. The tetramer positive populations indicate that the TILs contain a portion of cells that are reactive to the corresponding melanoma-associated antigens, through the HLA:A2:01 locus. Donors indicated with a * were utilized in the PDX efficacy study as depicted in FIG. 30.

[0038] **FIG. 28** shows interferon gamma (IFN γ) production after TIL:tumor cell co-culture to accurately predict TIL donors that are reactive to the PDX. This *in vitro* assay demonstrates that TIL donors 006, 39A, and 41A are the donors with the highest amount of IFN γ produced in response to the PDX, thus supporting their candidacy as donors to examine *in vivo* efficacy as described in Example 15.

[0039] **FIG. 29** is a schematic showing an exemplary melanoma patient-derived xenograft model treated with expanded TILs that express mbIL15 operably linked to a CA2 DRD and the CA2 ligand ACZ.

[0040] **FIG. 30** shows that treatment of patient-derived xenograft models according to the treatment paradigm shown in FIG. 29 results in superior anti-tumor efficacy as compared to treatment with an unengineered TIL and concomitant IL2 treatment. At the end of the end of the rapid expansion protocol (REP), unengineered TILs and regulated mbIL15 TILs (+/- acetazolamide (ACZ)) were adoptively transferred into mice bearing a human melanoma PDX. Mean tumor volumes were evaluated (+/- SEM).

[0041] **FIG. 31A-B** shows that TILs express mbIL15 operably linked to a CA2 DRD show significantly more intratumoral infiltration than unengineered TILs + IL2. **FIG. 31A** are photomicrographs of tumor sections stained immunohistochemically for human CD3 and showing intratumoral infiltration of TILs in animals treated with unengineered TILs and IL2, animals treated with TILs expressing mbIL15 operably linked to a CA2 DRD in the presence and absence of the CA2 ligand ACZ. **FIG. 31B** are graphs showing TIL numbers in stroma + tumor, stroma only, and tumor only.

DETAILED DESCRIPTION

[0042] Current processes for expanding TILs and T cells requires an interleukin 2 (IL2)-based TIL expansion (pre-rapid expansion protocol or pre-REP) followed by a rapid

expansion protocol (REP). During the pre-REP stage, TILs or T cells are cultured with exogenous IL2 and the presence of tumor antigens in the chunks of dissected tumor tissue. Thus, pre-REP requires IL2 in the absence of feeder cells. The REP step typically requires added feeder cells to support rapid TIL or T cell expansion. REP feeder cell and TIL or T cell stimulation are typically irradiated peripheral blood mononuclear cells (PBMCs) and high doses of IL2. The IL2 during REP, however, tends to exhaust the cells, resulting in a less potent TIL or T cell product. After *in vitro* REP using the current processes, expanded TILs or T cells are administered to the patient along with IL2, which may be given before, during, and/or after TIL/T cell administration, again pushing the cells to exhaustion. The current general protocol for TIL therapy requires high-dose IL2 administration beginning on the same day or the day after TIL infusion. By way of example, a high-dose IL2 regimen can consist of bolus intravenous infusions every eight hours until tolerance, for a maximum of 14 doses, nine days of rest, and a repeat for another 14 doses. Other IL2 regimens may consist of a four day cycle of IL2 administration that is repeated every 28 days for a maximum of four cycles or a PEGylated IL2 regimen that lasts up to 21 days.

[0043] In addition to promoting exhaustion of the TILs or T cells, high doses of IL2 can cause severe side effects in patients with cancer and often cannot be tolerated by those patients in need of ACT. The present compositions and methods provide a TIL or T cell therapy that optionally requires no exogenous cytokine administration, such as interleukins like IL2, before, during or after administration with of the TILs or T cells. Stated differently, with the present method, there is no need for concomitant interleukin therapy with TIL or T cell infusion. For example, optionally the subject does not require administration of exogenous IL2 preceding TIL or T cell infusion, or for 5 days, 7 days, 10 days, 14 days, 21 days, or 28 days after TIL infusion. Similarly, the present method eliminates the need for infusion of modified IL2 or other modified cytokine (such as a modified IL7 or IL15). By way of example, a modified interleukin can be a mutant or fragment of IL2, IL7, or IL15 that retains one or more functions of IL2, IL7, or IL15 but has reduced binding affinity to certain receptors, such as receptors that can promote CD4⁺ T_{reg} cell proliferation (e.g., by having reduced affinity).

[0044] As used herein, expansion refers to an increase in number or amount. When the term expansion is used herein in reference to a population or subpopulation of T cells or TILs, the term refers to a population of cells after REP. The size of the population (i.e., the number of T cells or TILs after REP) is greater than an unexpanded population (i.e., the number of T

cells or TILs pre-REP or the number of T cells or TILs after an unsuccessful REP resulting in the absence of a functional expansion of cells). When used in reference to a cell, such as an expanded T cell or expanded TIL, it refers to a T cell or TIL that has undergone and is the product or result of REP (i.e., a culture with feeder cells and selected stimulatory factors) that has resulted in functional expansion of the TIL population. Thus, as used herein an expanded T cell or TIL is progeny of a T cell or TIL (e.g., a modified T cell or modified TIL) cultured under REP resulting in functional expansion. Similarly, an unexpanded T cell or TIL as used herein refers to a T cell or TIL that has not yet undergone REP. Such an unexpanded T cell or TIL, however, may have gone through an initial IL2 pre-REP step or an unsuccessful REP resulting in the absence of a functional expansion of cells.

[0045] As used herein the term expansion can be used quantitatively, such as expands more, expands less, greater expansion, less expansion, and the like. Such relative terms generally refer to a greater to less fold increase in number of T cells or TILs in a population or subpopulation as compared to a different population or subpopulation (e.g., expansion of modified T cells or TILs as compared to expansion of an unmodified subpopulation). Thus, for example, a greater expansion of a subpopulation of modified T cells or TILs as compared to unmodified T cells or TILs means a greater fold increase, such as 1.5-fold as compared to a 1.25-fold increase, a 2-fold increase as compared to a 1.5-fold increase, a 5-fold increase as compared to a 2-fold increase, a 10-fold increase as compared to a 5-fold increase, a 40-fold increase as compared to a 10-fold increase, and the like of the modified T cells or TILs as compared to the unmodified T cells or TILs.

Methods for Expanding Modified T Cells or Tumor-Infiltrating Lymphocytes Using K562 Cells

[0046] Provided herein is a method of expanding modified T cells or TILs in the presence of engineered feeder cells. More particularly, a method is provided for expanding T cells or TILs engineered to express a cytokine (e.g., IL15), CAR, and/or TCR in the presence of modified K562 feeder cells, optionally in the absence of exogenous cytokine (e.g., an interleukin like IL2) during the REP stage.

[0047] The engineered feeder cells are K562 feeder cells, which can be modified to comprise a first exogenous nucleic acid sequence that encodes a costimulatory molecule chosen from the tumor necrosis factor superfamily and a second exogenous nucleic acid sequence encoding IL21 or IL7. Parental K562 feeder cells obtained from the American Type Culture

Collection (ATCC) can be modified by transduction with one or more heterologous polynucleotides. In some embodiments, the feeder cells are modified by introduction of the polypeptide by a viral or non-viral vector delivery method. As non-limiting examples, a vector comprising one or more heterologous polynucleotides may be introduced into a TIL or T cell by physical methods such as needles, electroporation, sonoporation, hydroporation; chemical carriers such as inorganic particles (e.g. calcium phosphate, silica, gold) and/or chemical methods. In some embodiments, synthetic or natural biodegradable agents may be used for delivery such as cationic lipids, lipid nano emulsions, nanoparticles, peptide-based vectors, or polymer-based vectors. By way of example, the feeder cells can be transduced by viral (e.g., retroviral or lentiviral) transduction with a nucleic acid encoding 41BBL (CD137L). The transduced feeder cells can be further transduced with a nucleic acid sequence that encodes mbIL21, e.g., with a Sleeping Beauty transposon expressing mbIL21. By way of example, the transposon can comprise a first nucleic acid sequence encoding IL21 and a second nucleic acid sequence encoding a transmembrane domain. The transmembrane domain optionally comprises an IL21 receptor, a MHC1 transmembrane domain, a CD8 transmembrane domain, a B7-1 transmembrane domain, a CD4 transmembrane domain, a CD28 transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, or a human IgG4 transmembrane domain. The IL21 can be directly linked to the transmembrane domain or may be connected via a linker or hinge. Thus, when the nucleic acid sequences are expressed, the IL21 is directly or indirectly linked to the transmembrane domain, which tethers the IL21 to the membrane of the transduced K562 cell. Optionally the feeder cells are not modified to express Fc- γ receptor CD32.

[0048] Numerous linker sequences (linkers) are known in the art. Linkers include, without limitations, GS linkers, GSG linkers, and GGSG linkers. These linkers are repeats of the subunit one or more times. Thus, a GS linker is a GS_n linker where n is a numerical number being 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more. Similarly, a GSG linker is a GS_n linker wherein n is a numerical number being 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. A GGSG linker is a GGSG_n linker where n is a numerical number being 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more.

[0049] A hinge sequence is a short sequence of amino acids that facilitates flexibility between connected components. The hinge sequence can be any suitable sequence derived or obtained from any suitable molecule. The hinge sequence may be derived from all or part of an immunoglobulin (e.g., IgG1, IgG2, IgG3, IgG4) hinge region, i.e., the sequence that falls between the CH1 and CH2 domains of an immunoglobulin (e.g., an IgG4 Fc hinge), or the

extracellular regions of type 1 membrane proteins such as CD8 α CD4, CD28 and CD7, which may be a wild type sequence or a derivative thereof. Some hinge regions include an immunoglobulin CH3 domain or both a CH3 domain and a CH2 domain. In some embodiments, the hinge is derived from a transmembrane domain.

[0050] In the event that the K562 feeder cells are not modified to express a costimulatory molecule chosen from the tumor necrosis factor superfamily and/or IL21 or IL7, exogenous costimulatory molecule and/or interleukin can be added to the culture medium. However, modifying the feeder cells to express these molecules eliminates or reduces the need for adding exogenous costimulatory molecules or interleukins during the REP stage.

[0051] The nucleic acid sequence that encodes the IL21 or IL7 or that encodes the 41BBL or both can include a signal sequence or leaders sequence. By way of example, the signal sequence can be a GM-CSF signal peptide (SEQ ID NO: 33).

[0052] Before feeder cells are used in the present methods, they are first rendered replication incompetent. Various means of treating the feeder cells are known in the art. Such methods include irradiation (e.g., with gamma rays), mitomycin-C treatment, electric pulses, mild chemical fixation (e.g., with formaldehyde or glutaraldehyde), or transduction of the feeder cells with a suicide gene. In some embodiments, the feeder cells are human cells. By way of example, the irradiation can be at 25-300 Gy delivered for example by a cesium source or an X-ray source.

[0053] Notably the method of expanding modified T cells or TILs can occur in the absence of exogenous cytokine (e.g., IL2) during the REP stage. The T cells or TILs expanded according to the methods described herein are modified. For example, the T cells or TILs can be modified to express mbIL15, a CAR or TCR. The expressed cytokine (e.g., mbIL15), CAR, or TCR can be operably linked to a DRD.

[0054] Expansion according to the present methods optionally results in at least a 10-fold expansion within 7 to 21 days, but can result in expansion greater than 40-fold, greater than 75 fold, or greater than 100-fold. For example, the modified T cells or TILs expand 500-2000 fold or any amount in between within 14 days. Fold expansion can be calculated by the number of cells of interest in culture at end of REP divided by number of cells of interest that were placed in culture at the beginning of REP.

Cell Cultures

[0055] Provided herein is a culture comprising modified T-cells or TILs and modified K562 feeder cells, wherein the K562 feeder cells comprise a first exogenous nucleic acid sequence encoding a costimulatory molecule chosen from the tumor necrosis factor superfamily and a second exogenous nucleic acid sequence encoding IL21 or IL7. T cells or TILs can be prepared from a subject and engineered to express a cytokine, a CAR, and/or a TCR. For example, the T cells or TIL can be engineered to express IL15 (e.g., mbIL15), which can be operably linked to a DRD.

[0056] The modified K562 feeder cells in the culture are replication incompetent and are engineered to express 41BBL and, optionally, IL21. Optionally, the modified K562 feeder cells express 41BBL and the IL21 is present in the culture medium. Optionally, the modified K562 feeder cells express 41BBL and mbIL21 or secreted IL21. IL21 polypeptide (e.g., UniProtKB-A0A224B028_HUMAN)). In one embodiment, the IL21 comprises the amino acid sequence corresponding to SEQ ID NO:35 or a polypeptide having at least 85, 90, 95, or 99% identity to SEQ ID NO:35 that retains one or more IL21 functions (e.g., promoting expansion of modified T cells or TILs *in vitro*). Optionally, the IL21 is directly or indirectly bound to a transmembrane domain, which tethers the IL21 to the membrane of the K562 feeder cells. IL21 is optionally SEQ ID NO:35. Optionally, the modified K562 feeder cells express 41BBL (e.g., SEQ ID NO:47). An exemplary IL21-41BBL insert is shown in Table 3. See e.g., SEQ ID NO:46 and SEQ ID NO:47 for the nucleic acid and amino acid sequences. Optionally the K562 cells are not modified to express Fc- γ receptor CD32.

[0057] The culture can further comprise additional components such as defined culture medium for conditions that allow rapid expansion of the modified T cells or TILs. Optionally, no exogenous cytokines are added to the culture. For example, the culture optionally comprises no exogenous IL2. Optionally the initial ratio of feeder cells to T cells or TILs ranges from 1:1 to 200:1 or any ratio in between. In some embodiments, the initial ratio of feeder cells to TILs is 5:1. Upon completion of the REP step, however, the number of expanded T cells or TILs substantially exceeds the number of feeder cells, especially when replication incompetent feeder cells are used.

Modified Tumor Infiltrating Lymphocytes (TILs)

[0058] TILs include T cells, NK cells, B cells, and NKT cells, but, at least in certain tumor types, comprise predominantly T cells (e.g., cytotoxic T cells that are CD8⁺ and helper T

cells that are CD4+). The TILs described herein are engineered to express mbIL15. Thus, the modified TILs comprise an exogenous nucleic acid sequence that encodes IL15, an exogenous nucleic acid sequence that encodes a transmembrane domain, and, optionally, an exogenous nucleic acid sequence that encodes a linker or hinge. IL15 is not generally expressed as a membrane bound molecule, thus, to express mbIL15, the IL15 must be associated with a transmembrane domain. IL15 as used herein refers to an IL15 polypeptide (e.g., UniProtKB - P40933 (IL15_HUMAN)). In one embodiment, the IL15 payload comprises the amino acid sequence provided in Table 2 (SEQ ID NO:12) or a polypeptide having at least 85, 90, 95, or 99% identity to SEQ ID NO:12 that retains one or more IL15 functions (e.g., promoting expansion of modified TILs *in vivo*, promoting cytotoxicity of T and NK cells).

[0059] Exemplary transmembrane domains include a MHC1 transmembrane domain, a CD8 transmembrane domain, a B7-1 (CD80) transmembrane domain, a CD4 transmembrane domain, a CD28 transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, a human IgG4 transmembrane domain, or an IL15 receptor subunit (e.g., IL15 α R). The IL15 can be directly linked to the transmembrane domain or may be connected via a linker or hinge.

[0060] The modified TILs described herein optionally further comprise an exogenous nucleic acid sequence that encodes an intracellular (cytoplasmic) tail. The intracellular tail can be, for example, a B7-1 (CD80) intracellular tail.

[0061] The modified TILs described herein optionally further comprises an exogenous nucleic acid sequence that encodes a signal sequence (leader sequence). Exemplary leader sequences include MDMRVPAQLLGLLLWLSGARC (SEQ ID NO:10), MDWTWILFLVAAATRVHS (IgEss; SEQ ID NO:58), MRISKPHLRSISIQCYLCLLLNSHFLTEAGIHVFILGCF SAGLPKTEA (Native IL15 LS; SEQ ID NO:59), MGLVRRGARAGPRMPRGWTALCLLSLLPSGFMA (CD34; SEQ ID NO:60).

[0062] Additionally, certain TILs further comprise an exogenous nucleic acid sequence that encodes a DRD. IL15 is important for T cell and NK cell proliferation, but continuous exposure to high levels of IL15 may lead to exhaustion of these cells *in vivo*, which would decrease the efficacy of IL15 expressing TILs. Thus, in certain embodiments, a DRD is operably linked to the mbIL15 to provide regulation of the IL15 activity or abundance during TIL immunotherapy.

[0063] Drug responsive domains (DRDs) are polypeptides that regulate the expression or activity level of a payload. Although referred to as drug responsive domains, the ligand to which a DRD is responsive need not be a drug. More specifically, DRDs interact with a ligand such that, when the DRD is operatively linked to a payload, it confers ligand-dependent reversible regulation of a characteristic of the payload (for example, activity or expression level). U.S. Pat. Nos. 9,487,787 and 10,137,180, U.S. Publication Nos.: 2019/0192691; 2020/0101142; 2020/0172879; 2021/0069248, and U.S. Pat. App. Nos.: 17,251,635; and 17/288,373, the contents of each of which are hereby incorporated by reference in their entirety, provide examples of DRDs (and their paired ligands) according to this disclosure. Certain of these and other example DRDs according to this disclosure are also provided elsewhere in this specification. The DRDs, by way of example, can be chosen from FKBP (SEQ ID NO:4), ecDHFR (SEQ ID NO:1), hDHFR (SEQ ID NO:2), ER (SEQ ID NO:9), PDE5 full length (SEQ ID NO:6), PDE5 ligand binding domain (SEQ ID NO:5) and CA2 (SEQ ID NO:7) or a portion of any of the foregoing that maintains DRD function or an amino acid sequence having at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NOs: 1, 2, 4, 5, 6, 7, or 9 or the DRD functional portion thereof. One or more mutations (including truncations, substitutions, and deletions) in the amino acid sequence of FKBP, ecDHFR, hDHFR, ER, PDE5, and CA2, for example, can be advantageous to further destabilize the DRD. Suitable DRDs, which may be referred to as destabilizing domains or ligand binding domains, are also known in the art. See, e.g., WO2018/161000; WO2018/231759; WO2019/241315; US8,173,792; US8,530,636; WO2018/237323; WO2017/181119; US2017/0114346; US2019/0300864; WO2017/156238; Miyazaki et al., J Am Chem Soc, 134:3942 (2012); Banaszynski et al. (2006) Cell 126:995-1004; Stankunas, K. et al. (2003) Mol. Cell 12:1615-1624; Banaszynski et al. (2008) Nat. Med. 14:1123-1127; Iwamoto et al. (2010) Chem. Biol. 17:981-988; Armstrong et al. (2007) Nat. Methods 4:1007-1009; Madeira da Silva et al. (2009) Proc. Natl. Acad. Sci. USA 106:7583-7588; Pruett-Miller et al. (2009) PLoS Genet. 5:e1000376; and Feng et al. (2015) Elife 4:e10606, the contents of each of which are hereby incorporated by reference in their entirety.

[0064] Without meaning to be limited by theory, DRDs are thought to be unstable polypeptides that degrade in the absence of their corresponding stabilizing ligand (also referred to as the paired ligand or ligand), but whose stability is rescued by binding to the stabilizing ligand. Because binding of the ligand to the DRD is reversible, later removal of

the ligand results in the DRD unfolding, becoming unstable, and ultimately being tagged for degradation by the ubiquitin-proteasome system (“UPS”). Accordingly, it is believed that when a DRD is operably linked to a payload like mbIL15, the entire construct (i.e., DRD plus IL15) itself is rendered unstable and degraded by the UPS. However, in the presence of the paired ligand, the construct is stabilized and the mbIL15 payload remains available. Further, it is believed that the conditional nature of DRD stability allows a rapid and non-perturbing switch from stable protein to unstable UPS substrate and may facilitate regulation or modulation of a payload’s activity level, and/or modulation of a payload’s activity level.

[0065] Because the abundance of a payload (e.g., mbIL15) is related to the activity of a payload, level of abundance and level of activity or abundance and activity are referred to herein as activity or activity level unless explicitly stated otherwise or nonsensical in context. Further, measurements of abundance are used as a proxy for activity level and may be used herein to reflect the activity level. Consequently changes in the abundance of a payload in the presence of an effective amount of ligand as compared to the absence of ligand optionally serves as a proxy for measuring changes in activity level. Accordingly, level of abundance and activity level are used interchangeably throughout this disclosure.

[0066] Numerous DRD are described herein, but one of skill in the art could identify additional DRDs. By way of example, DRDs can be identified using library screening and structure-guided engineering to select the optimal DRD variant with sufficient instability in the absence of the ligand and sufficient stability in the presence of the ligand. A variant library can be generated using random mutagenesis screening by transducing cells (e.g., Jurkat cells) with mutant DRD candidates. To produce an enriched library, cells with the desired characteristics (low basal activity/expression and high dynamic range) are then selected by testing the expression of a report gene across a range of concentrations of ligand. Single cell clones are then produced and characterized to identify candidate DRDs. The DRD is capable of affecting a characteristic, for example, the abundance or activity level, of a payload to which it is operably linked. Further, the one or more DRDs interact with a ligand to provide ligand-dependent reversible regulation of the characteristic of the payload.

[0067] The DRDs described herein are responsive to a paired ligand. Optionally, the DRDs are responsive to a paired ligand that is a small molecule drug, such as an FDA-approved small molecule. However, one of skill in the art can select the DRD and its paired ligand to meet the specific needs of the system. Examples of stabilizing ligands and their uses for specific DRDs described herein are shown in Table 1 and in U.S. Patent No. 9,487,787 filed

March 33, 2012, U.S. Patent No. 10,137,180 filed September 6, 2013, PCT Application No. PCT/US2018/037005, filed June 12, 2018, PCT Application No. PCT/US2019/036654 filed June 12, 2019, PCT Application No. PCT/US2019/057698 filed October 23, 2019, PCT Application No. PCT/US2020/021596 filed March 6, 2020, and U.S. Application No. 16/558,224 filed September 2, 2019, the disclosures of all of the aforereferenced applications are incorporated herein by reference in their entireties.

Table 1. Listing of DRD and exemplary ligands

DRD Protein	SEQ ID NO:	Exemplary Ligands
<i>E. coli</i> dihydrofolate reductase (ecDHFR) (Uniprot ID: P0ABQ4)	1	Methotrexate (MTX) Trimethoprim (TMP)
Human dihydrofolate reductase (hDHFR) (Uniprot ID: P00374)	2	Methotrexate (MTX) Trimethoprim (TMP)
Human FKBP (FK506 binding protein) (Uniprot ID: P62942)	4	Shield-1
Phosphodiesterase 5 (PDE5), ligand binding domain (Uniprot ID: Uniprot ID O76074)	5	Sildenafil; Vardenafil; Tadalafil
Phosphodiesterase 5 (PDE5), full-length (Uniprot ID: Uniprot ID O76074)	6	Sildenafil; Vardenafil; Tadalafil
Carbonic anhydrase II (CA2) (Uniprot ID: P00918)	7	Celecoxib Acetazolamide
Human estrogen receptor (ER) (Uniprot ID: P03372.2)	9	Bazedoxifene Raloxifene

[0068] Optionally, a DRD of the present disclosure may be derived from human carbonic anhydrase 2 (hCA2), which is a member of the carbonic anhydrases, a superfamily of metalloenzymes. A DRD of the present disclosure may be derived from amino acids 1-260 of CA2 (Uniprot ID: P00918) (SEQ ID NO:7). Optionally, DRDs are derived from CA2 comprising amino acids 2-260 of the parent CA2 sequence (e.g., amino acids 2-260). This is referred to herein as a CA2 M1del mutation (CA2; SEQ ID NO:55). Optionally, a DRD of the present disclosure comprises a region of or the whole human carbonic anhydrase 2, and further comprises one or more mutations relative to the full length sequence selected from M1del, L156H, and S56N. Optionally, the DRD is selected from the group consisting of SEQ ID NOs:7, 26, 55, 56, and 57.

[0069] By way of example, the modified TIL can comprise a nucleic acid sequence that encodes a transmembrane domain that is C-terminal to the IL15 polypeptide component and an intracellular tail that is C-terminal to the transmembrane domain.

[0070] Non-limiting examples of constructs and construct components for the modified TILs are shown in Table 2. The construct designated OT-IL15-292 includes from the N terminus a signal sequence, IL15, (GS)₁₅ linker, a hinge region, a transmembrane region, and an intracellular tail. The construct designated OT-IL15-293 includes a DRD (specifically, a CA2 DRD (M1del, L156H)) at the C terminus.

Table 2: Examples of constructs and construct components

Description	Amino Acid Sequence (AA)	Nucleic Acid Sequence (NA)	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
Leader sequence	MDMRVPAQLLGL LLLWLSGARC	ATGGACATGCGGGTGCCTGCACAACTT CTGGGCCTGCTGTTGTTGTGGCTGTCTG GAGCCCGGTGT	10	11
Interleukin-15 (IL15)	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVT AMKCFLELQVIS LESGDASIHDVE NLIHLANNSLSSN GNVTESGCKECE ELEEKNIKEFLQS FVHIVQMFINTS	AATTGGGTAAATGTTATCAGTGATCTC AAGAAGATAGAGGATCTCATCCAGTCC ATGCATATTGATGCCACGCTGTACACA GAAAGCGATGTGCATCCTAGCTGTAAG GTGACAGCGATGAAGTGTCTTTCTTTTGG AGCTGCAGGTAATTAGTCTTGAGTCCG GCGATGCCAGCATTTCATGATACCGTAG AAAACCTTGATTATCCTGGCCAACAATT CTCTGTCCTCAAACGGAAACGTAACCG AGAGCGGTTGTAAAGAATGTGAAGAAC TGGAAGAAAAGAACATCAAGGAGTTTC TGCAATCATTCGTTACATCGTACAAAT GTTCATAAATACGTCA	12	13
Linker (GS) ₁₅	GSGSGSGSGSGSG SGSGSGSGSGSGS GSGS	GGATCTGGTTCTGGTTCCGGAAGTGGA TCTGGTTCAGGGTCCGGTAGTGGATCT GGGTCAGGAAGTGGAAGCGGTAGTGG GTCTGGATCT	14	15
Hinge	KQEHFPDN	AAACAAGAGCACTTTCCTGATAAC	16	17
Transmembrane	LLPSWAITLISVN GIFVICCL	CTGTTGCCGAGCTGGGCGATTACGCTT ATCAGTGTAACGGCATCTTTGTAATAT GCTGTCTG	18	19

Description	Amino Acid Sequence (AA)	Nucleic Acid Sequence (NA)	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
Intracellular tail	TYCFAPRCRERRR NERLRRESVRPV	ACCTACTGCTTCGCACCAAGGTGCCGG GAGAGAAGGAGAAATGAAAGACTGAG AAGGGAGAGCGTGAGACCTGTG	20	21
Intracellular tail	TYCFAPRCRERA RNERLRRETVP V	ACCTACTGCTTCGCACCAAGGTGCCGG GAGAGAGCAAGAAATGAAAGACTGAG AAGGGAGACCGTGAGACCTGTG	22	23
Linker (GS)	GS	GGATCC	24	25
Drug Responsive Domain (CA2 (M1del, L156H))	SHHWGYGKHNG PEHWHKDFPIAK GERQSPVDIDTHT AKYDPSLKPLSVS YDQATSLRILNNG HAFNVEFDDSQD KAVLKGGPLDGT YRLIQFHFHWGSL DGQGSEHTVDKK DSIKTKGKSADFT NFDPRGLLPESLD YWTPGSLTTPPL LECVTWIVLKEPI SVSSEQVLKFRKL NFNGEGEPEELM VDNWRPAQPLKN RQIKASFK	TCCCATCACTGGGGGTACGGCAAACAC AACGGACCTGAGCACTGGCATAAGGAC TTCCCCATTGCCAAGGGAGAGCGCCAG TCCCCTGTTGACATCGACACTCATAACAG CCAAGTATGACCCTTCCCTGAAGCCCCT GTCTGTTTCCTATGATCAAGCAACTTCC CTGAGAATCCTCAACAATGGTCATGCT TTCAACGTGGAGTTTGATGACTCTCAG GACAAAGCAGTGCTCAAGGGAGGACCC CTGGATGGCACTTACAGATTGATTGAG TTTCACTTTCACTGGGGTTCACTTGATG GACAAGGTTGAGAGCATACTGTGGATA AAAAGAAATATGCTGCAGAACTTCACT TGGTTCCTGGAACACCAAATATGGGG ATTTTGGGAAAGCTGTGCAGCAACCTG ATGGACTGGCCGTTCTAGGTATTTTTTT GAAGGTTGGCAGCGCTAAACCGGGCCA TCAGAAAGTTGTTGATGTGCTGGATTCC ATTAAAACAAAGGGCAAGAGTGCTGAC TTCCTAACTTCGATCCTCGTGGCCTCC TTCCTGAATCCCTGGATTACTGGACCTA CCCAGGCTCACTGACCACCCCTCCTCTT CTGGAATGTGTGACCTGGATTGTGCTC AAGGAACCCATCAGCGTCAGCAGCGAG CAGGTGTTGAAATTCCGTAAACTTAAC TTCAATGGGGAGGGTGAACCCGAAGAA	26	27

Description	Amino Acid Sequence (AA)	Nucleic Acid Sequence (NA)	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
		CTGATGGTGGACAACCTGGCGCCCAGCT CAGCCACTGAAGAACAGGCAAATCAAA GCTTCCTTCAAA		
OT-IL15-292	MDMRVPAQLLGL LLLWLSGARCNW VNVISDLKKIEDLI QSMHIDATLYTES DVHPSCCKVTAMK CFLLELQVISLES GDASIHDTVENLII LANNSLSSNGNV TESGCKECEEELE KNIKEFLQSFVHI VQMFINTSGSGSG SGSGSGSGSGSGS SGSGSGSGSGSGSK QEHFPDNLLPSW AITLISVNGIFVIC CLTYCFAPRCRER RRNERLRRESVRP VGS	ATGGACATGCGGGTGCCTGCACAACCTT CTGGGCCTGCTGTTGTTGTGGCTGTCTG GAGCCCGGTGTAATTGGGTAAATGTTA TCAGTGATCTCAAGAAGATAGAGGATC TCATCCAGTCCATGCATATTGATGCCAC GCTGTACACAGAAAGCGATGTGCATCC TAGCTGTAAGGTGACAGCGATGAAGTG TTTTCTTTTGGAGCTGCAGGTAATTAGT CTTGAGTCCGGCGATGCCAGCATTCAT GATACCGTAGAAAACCTTGATTATCCTG GCCAACAATTCTCTGTCTCAAACGGA AACGTAACCGAGAGCGGTTGTAAAGAA TGTGAAGAACTGGAAGAAAAGAACATC AAGGAGTTTCTGCAATCATTCGTTTACA TCGTACAAATGTTTATAAATACGTCAG GATCTGGTTCTGGTTCCGGAAGTGGAT CTGGTTCAGGGTCCGGTAGTGGATCTG GGTCAGGAAGTGGGAAGCGGTAGTGGGT CTGGATCTAAACAAGAGCACTTTTCTG ATAACCTGTTGCCGAGCTGGGCGATTA CGCTTATCAGTGTAACCGGCATCTTTGT AATATGCTGTCTGACCTACTGCTTCGCA CCAAGGTGCCGGGAGAGAAGGAGAAA TGAAAGACTGAGAAGGGAGAGCGTGA GACCTGTGGGATCC	28	29
OT-IL15-293	MDMRVPAQLLGL LLLWLSGARCNW VNVISDLKKIEDLI QSMHIDATLYTES DVHPSCCKVTAMK	ATGGACATGCGGGTGCCTGCACAACCTT CTGGGCCTGCTGTTGTTGTGGCTGTCTG GAGCCCGGTGTAATTGGGTAAATGTTA TCAGTGATCTCAAGAAGATAGAGGATC TCATCCAGTCCATGCATATTGATGCCAC	30	31

Description	Amino Acid Sequence (AA)	Nucleic Acid Sequence (NA)	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
	CFLLELQVISLES GDASIHDTVENLII LANNSLSSNGNV TESGCKECEEELEE KNIKEFLQSFVHI VQMFINTSGSGSG SGSGSGSGSGSGS GSGSGSGSGSGSK QEHFPDNLPSW AITLISVNGIFVIC CLTYCFAPRCRER RRNERLRRESVRP VGSSHHWGYGK HNGPEHWHKDFP IAKGERQSPVDID THTAKYDPSLKPL SVSYDQATSLRIL NNGHAFNVEFDD SQDKAVLKGGPL DGTYRLIQFHH WGLDQGQSEHT VDKKDSIKTKGK SADFTNFDPRGLL PESLDYWTYPGS LTTPLLECVTWI VLKEPISVSSEQV LKFRKLNFNNEG EPEELMVDNWRP AQPLKNRQIKASF K	GCTGTACACAGAAAGCGATGTGCATCC TAGCTGTAAGGTGACAGCGATGAAGTG TTTTCTTTTGGAGCTGCAGGTAATTAGT CTTGAGTCCGGCGATGCCAGCATTCAT GATACCGTAGAAAACCTTGATTATCCTG GCCAACAAATTCTCTGTCTCTCAAACGGA AACGTAACCGAGAGCGGTTGTAAAGAA TGTGAAGAACTGGAAGAAAAGAACATC AAGGAGTTTCTGCAATCATTTCGTTTACA TCGTACAAATGTTTCATAAATACGTCAG GATCTGGTTCTGGTTCCGGAAGTGGAT CTGGTTCAGGGTCCGGTAGTGGATCTG GGTCAGGAAGTGGAAGCGGTAGTGGGT CTGGATCTAAACAAGAGCACTTTTCCTG ATAACCTGTTGCCGAGCTGGGCGATTA CGCTTATCAGTGTAACCGGCATCTTTGT AATATGCTGTCTGACCTACTGCTTCGCA CCAAGGTGCCGGGAGAGAAGGAGAAA TGAAAGACTGAGAAGGGAGAGCGTGA GACCTGTGGGATCCTCCCATCACTGGG GGTACGGCAAACACAACGGACCTGAGC ACTGGCATAAGGACTTCCCCATTGCCA AGGGAGAGCGCCAGTCCCCTGTTGACA TCGACACTCATACAGCCAAGTATGACC CTTCCCTGAAGCCCCTGTCTGTTTCCTA TGATCAAGCAACTTCCCTGAGAATCCT CAACAATGGTCATGCTTTCAACGTGGA GTTTGATGACTCTCAGGACAAAGCAGT GCTCAAGGGAGGACCCCTGGATGGCAC TTACAGATTGATTCAGTTTCACTTTCAC TGGGGTTCACTTGATGGACAAGGTTCA GAGCATACTGTGGATAAAAAGAAATAT GCTGCAGAACTTCACTTGGTTCACTGG		

Description	Amino Acid Sequence (AA)	Nucleic Acid Sequence (NA)	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
		AACACCAAATATGGGGATTTTGGGAAA GCTGTGCAGCAACCTGATGGACTGGCC GTTCTAGGTATTTTTTTGAAGGTGGCA GCGCTAAACCGGGCCATCAGAAAGTTG TTGATGTGCTGGATTCCATTA AAAACAA AGGGCAAGAGTGCTGACTTCACTAACT TCGATCCTCGTGGCCTCCTTCCTGAATC CCTGGATTACTGGACCTACCCAGGCTC ACTGACCACCCCTCCTCTTCTGGAATGT GTGACCTGGATTGTGCTCAAGGAACCC ATCAGCGTCAGCAGCGAGCAGGTGTTG AAATTCCGTAAACTTAACTTCAATGGG GAGGGTGAACCCGAAGAACTGATGGTG GACAACTGGCGCCCAGCTCAGCCACTG AAGAACAGGCAAATCAAAGCTTCCTTC AAA		

[0071] To create a membrane-tethered cytokine like IL15 operably linked to a DRD with a sufficient dynamic range (i.e., an acceptable activity range that corresponds to variation in ligand from zero to maximum saturation), the polypeptide optionally includes from the N-terminus the payload (IL15), a linker, a hinge, a transmembrane region, a tail, and a DRD. The tail and/or linker and tail and linker length may influence activity in the absence of ligand and in some embodiments, the specific tail and/or linker and length are chosen to maximize the on-state (e.g., maximum activity level) while maintaining low basal activity level and ligand responsiveness. The specific hinge may allow for conformation changes and thereby influence ligand responsiveness across a sufficient dynamic range.

[0072] The modified TILs that express mbIL15 as described herein have a number of advantages. First, the modified TILs can be expanded *in vitro* in the presence of feeder cells (such as K562 feeder cells that express 41BBL, and IL21). Significantly, subsequent to the pre-REP stage, the modified TILs can expand *in vitro* in the absence of exogenous cytokine and the expanded TILs are activated and can expand further *in vivo* without administration of an exogenous cytokine, such as IL2.

[0073] A population of TILs comprising a plurality of modified TILs can include a subpopulation of the TILs that has undergone expansion (i.e., REP with feeder cells and stimulatory molecules, such as IL12 and 4BB1). The expanded TILs demonstrate a number of advantages. For example, expanded TILs that have undergone REP are then capable of surviving in a culture lacking feeder cells. More particularly, TILs engineered to express mbIL15 can survive longer than unengineered cells in the absence of an exogenous cytokine, for example, an interleukin such as IL2. TILs engineered to express mbIL15 operably linked to a DRD, survive better in the presence of the ligand but in the absence of exogenous cytokine than unengineered TILs or regulated mbIL15 TILs in the absence of ligand. Additionally, a population of expanded TILs shows preferential expansion of certain TILs and, thus, fewer or more subtypes of TILs as compared to a control population of unexpanded TILs. A control population of unexpanded TILs as used herein refers to TILs that are similarly modified as the expanded TILs but that have not undergone REP.

[0074] A population of expanded TILs, for example, has a greater proportion of CD8⁺ cells, a lesser proportion of CD4⁺ cells, and a lower CD4⁺:CD8⁺ ratio as compared to a control population of unexpanded TILs. CD8⁺ TILs are considered key players in killing cancer cells by releasing cytotoxic molecules and cytokines, and the number of CD8⁺ TILs compared to the number of CD4⁺ TILs (i.e., the CD4⁺:CD8⁺ ratio) in a tumor has been found to correlate with a positive outcome.

[0075] Additionally, in certain embodiments, the population of expanded TILs has a lesser proportion of CD4 T_{reg} cells as compared to the proportion of Treg cells in the pre-REP TILs, prior to engineering and expansion in REP. CD4 T_{reg} cells have a role in immunological tolerance and immune homeostasis by suppressing immune reactions. Thus, a lower proportion of T_{reg} cells is desirable in immunotherapy, such as ACT with TILs.

[0076] The population of expanded TILs also shows fewer exhausted TILs and more polyfunctional TILs. The population of expanded TILs a lesser proportion of PD1⁺ cells as compared to the proportion of PD1⁺ cells in a control population of unexpanded TILs. Additionally, the population of expanded TILs has a greater proportion of cells producing both tumor necrosis factor α (TNF α) and interferon γ (IFN γ) as compared to the proportion of TILs producing both TNF α and IFN γ in a control population of unexpanded TILs.

[0077] Provided herein is a mixed population of TILs comprising a subpopulation of unmodified TILs and a subpopulation of modified TILs comprising a mbIL15, which is optionally operably linked to a DRD. The subpopulation of modified TILs expands in the

presence K562 feeder cells, 41BBL, and IL21. The subpopulation of modified TILs expands more than the subpopulation of unengineered TILs in the presence of K562 feeder cells, 41BBL, and IL21. This preferential expansion of modified TILs occurs in the absence of exogenous IL2 during the REP.

Modified T Cells

[0078] Modified T cells that can be expanded according to the methods described herein include, by way of example, activated T cells or T cells engineered to express a cytokine, a CAR, or a TCR. Methods of activation and methods of engineering T cells are known in the art. T cells can be engineered to express a cytokine, such as IL15 or, more specifically, a mbIL15 according to the methods described above for TILs. T cells can be engineered to express a CAR or TCR and, optionally, a cytokine (such as a membrane bound cytokine).

[0079] CARs comprising an extracellular targeting domain (e.g., a scFv that recognizes a specific tumor antigen or other tumor cell-surface molecules), a transmembrane domain/region, and an intracellular signaling/activation domain (e.g., the signal region of CD3 ζ , and/or one or more costimulatory signaling domains, such as those from CD28, 4-1BB (CD137) and OX-40 (CD134)) can be expressed by the modified T cells.

Methods of Making Modified TILs

[0080] TILs can be prepared from a tumor or a biopsy thereof using methods known in the art. For example, pieces of the tumor (e.g., 1-5 mm in size) are subjected to enzymatic digestion (e.g., collagenase (e.g., 0.5-5 mg/mL), DNase, hyaluronidase) or mechanical dissociation. The dissociated cells are incubated in cell culture media under conditions that favor the proliferation of TILs over other cells (i.e., in the presence of IL2). This stage is the pre-REP stage. In the pre-REP stage, the cells can be cultured (e.g., 3 to 28 days) in the presence of 2000-8000 IU/mL IL2 (e.g., 6000 IU/ml) and optionally in the presence of inactivated human AB serum. In some embodiments, the cells are cultured for a period of days, generally from 3 to 28 days. In some embodiments, this pre-REP cell population is cultured for a period of 7 to 21 days.

[0081] The pre-REP TILs or T cells can be cryopreserved. Cryopreserved cells are thawed and rested before activation. The cells can be activated using, for example, plate bound OKT3, soluble OKT3, costimulatory antibodies (e.g., antibodies to CD28 or 41BB) +OKT3,

anti-CD3 and anti-CD28 antibodies bound to bead or fragments, etc. The activation step can be 1-2 days or longer. Following activation, one or more TILs are then engineered to express a membrane-bound interleukin 15 (mbIL15) by transducing the one or more TILs with a vector having a first nucleic acid sequence that encodes IL15 and a second nucleic acid sequence that encodes a transmembrane domain. Optionally the vector further comprises one or more nucleic acid sequences that encode a signal peptide, a linker, a hinge, an intracellular tail, or a DRD. The vector can be configured any number of ways to achieve the desired mbIL15. Exemplary nucleic acid constructs include the nucleic acid sequences encoding OT-IL15-293 and OT-IL15-292, with and without DRDs, respectively. Thus, the vector optionally comprises SEQ ID NO:29, 31, 53 or 54. The vector includes or encode additional elements, such as a promoter sequence and other regulatory elements (enhancers, translational control elements (e.g., IRES), and elements that control half-life.)). The vector optionally comprises or can comprise nucleic acid sequences that encode elements that control translation (e.g., IRES, WPRE, and the like).

[0082] The vector can be chosen from viral vectors, plasmids, cosmids, and artificial chromosomes. By way of example, the vector can be a viral vector, such as a lentiviral vector or a retroviral vector. By way of example the viral vector can be a baboon endogenous retrovirus envelope (BaEV) pseudotyped lentiviral vector or a gibbon ape leukemia virus (GALV) envelop pseudotyped gamma-retroviral vector that comprises a nucleic acid sequence that encodes IL15 and a nucleic acid sequence that encodes a transmembrane domain. Upon expression, the IL15 is associated with the transmembrane domain and is membrane bound by the transmembrane domain.

[0083] Vectors are optionally transferred to cells by non-viral methods such as needles, electroporation, sonoporation, hydroporation, chemical carriers (such as inorganic particles (e.g. calcium phosphate, silica, gold)), and/or chemical methods. In some embodiments, synthetic or natural biodegradable agents are used for delivery such as cationic lipids, lipid nano emulsions, nanoparticles, peptide-based vectors, or polymer-based vectors.

[0084] The nucleic acid sequence that encodes IL15 can be genomic or non-genomic nucleic acid. That is, the delivery system used to deliver the IL15 encoding nucleic acid can be integrated into the genome of the TIL or can be non-integrated (i.e., episomal) or transferred as RNA into the cytoplasm using RNA vectors.

[0085] The TIL comprising mbIL15 are expanded in the REP stage (e.g., 5-21 days or any amount in between, including 7-14 days) without IL2. As described further in the Examples,

the TILs modified to express IL15 are expanded in the presence of K562 feeder cells as well as 41BBL and IL21. In certain embodiments, the K562 feeder cells are engineered to express 41BBL and IL21, which can be membrane bound IL21 (mbIL21), thus reducing or eliminating the need for exogenous cytokines such as IL2, IL7, or IL15 during the REP. In some embodiments, the modified TILs are cultured with the K562 cells modified to express mbIL21 and 41BBL at a ratio of 1:1 to 100:1, 1:1 to 50:1, 1:1 to 20:1, 1:1 to 10:1, or 2:1 to 5:1(TIL: feeder cell).

[0086] Before feeder cells are used in the present method, they are first rendered replication incompetent. Various means of treating the feeder cells are known in the art. Such methods include irradiation (e.g., with gamma or X-rays), mitomycin-C treatment, electric pulses, mild chemical fixation (e.g., with formaldehyde or glutaraldehyde), or transduction of the feeder cells with a suicide gene. In some embodiments, the feeder cells are human cells. By way of example, the irradiation can be at 25-100 Gy delivered for example by a cesium source or an X-ray source.

[0087] Following expansion on feeder cells, the TILs are optionally isolated from the feeder cells. As used herein, the term isolation is not meant to suggest that the TILs are entirely free of other components, such as feeder cells, just to suggest that the TILs are relatively free of feeder cells.

[0088] Provided herein is a method of making a TIL, a population of TILs, or a subpopulation of TILs that comprise mbIL15. Also provided are nucleic acid sequences encoding the mbIL15, vectors comprising the nucleic acid sequence encoding the mbIL15, replication incompetent K562 feeder cells that are modified to express 41BBL and IL21, and TILs made by the method described herein.

[0089] Provided herein is a method of making a TIL, a population of TILs, or a subpopulation of TILs that comprise mbIL15. Also provided are nucleic acid sequences encoding the mbIL15, vectors comprising the nucleic acid sequence encoding the mbIL15, replication incompetent K562 feeder cells that are modified to express 41BBL and IL21, and TILs made by the method described herein.

Methods of Making Modified T Cells

[0090] Primary human T cells can be derived from human peripheral blood mononuclear cells (PBMC), bone marrow, or umbilical cord and can be collected after stimulation with G-CSF. Nucleic acid constructs designed for cistronic or multicistronic expression are

introduced into the T cells using any delivery technique as described above. Optionally, the T cells are transduced with a cytokine, such as a membrane bound cytokine or both a cytokine and a receptor (e.g., a CAR or a TCR). For example, the T cells can be transduced with one or more nucleic acids encoding IL15, which, when expressed, is linked to a transmembrane domain; a CAR; or a TCR. By way of example, the T cell can be transduced with a first vector that encodes mbIL15 and a second vector that encodes one or more components of a CAR, such as an extracellular targeting domain (e.g., a scFv that recognizes a specific tumor antigen or other tumor cell-surface molecules), a transmembrane domain/region, and an intracellular signaling/activation domain (e.g., the signal region of CD3 ζ , and/or one or more costimulatory signaling domains, such as those from CD28, 4-1BB (CD137) and OX-40 (CD134)). Optionally, the T cell is transduced with a third vector that encodes any components of the CAR not encoded by the second vector.

Pharmaceutical Compositions

[0091] Provided herein is a pharmaceutical composition suitable for use in ACT. The pharmaceutical composition can comprise modified T cells or TILs, such as expanded T cells or TILs, and a pharmaceutically acceptable carrier. The population of modified T cells or TILs in the pharmaceutical composition is optionally a mixed population comprising a subpopulation of modified T cells (e.g., CAR⁺ T cells) or modified TILs (e.g., TILs engineered to express mbIL15) and unmodified T cells or TILs (i.e., unengineered T cells or TILs).

[0092] The term carrier means a compound, composition, substance, or structure that, when in combination with a compound or cells, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or cells for its intended use or purpose. For example, a carrier can be selected to minimize any degradation of the TILs and to minimize any adverse side effects in the subject. Such pharmaceutically acceptable carriers include sterile biocompatible pharmaceutical carriers, including, but not limited to, saline, buffered saline, artificial cerebral spinal fluid, dextrose, and water. By pharmaceutically acceptable is meant a material that is not biologically or otherwise undesirable, which can be administered to an individual along with the T cell or TIL population without causing unacceptable biological effects or interacting in a deleterious manner with the T cells or TILs.

[0093] Optionally the pharmaceutical composition further comprises a cryoprotectant (cryopreservant). Such a cryoprotectant serves to prevent unacceptable cell lysis or damage should the TILs be frozen for future use. Cryoprotectants are known in the art. Such cryoprotectants can be selected from among glycerol, ethylene glycol, propylene glycol, or dimethylsulfoxide (DMSO).

[0094] The pharmaceutical compositions described herein optionally further comprise one or more pharmaceutically acceptable excipients (e.g., human serum albumin or polymeric materials (e.g., PEG)).

[0095] The compositions of the present disclosure can be formulated in any manner suitable for delivery. The T cells or TILs, for example, can be administered in nanoparticles, poly (lactic-co-glycolic acid) (PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids, or combinations thereof.

[0096] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions suitable for administration to humans, it should be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human mammals. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, agricultural animals, such as cattle, horses, chickens and pigs; domestic animals, such as cats, dogs; or research animals such as mice, rats, rabbits, dogs and non-human primates.

Methods of Treatment

[0097] Provided herein are methods of treating cancer in a subject by administering to the subject (i.e., the recipient subject) a modified T cell or TIL population that has been expanded according to the methods described herein. Thus, modified T cells or TILs expanded in the presence of K562 cells, for example, K562 cells modified to express a costimulatory molecule of the TNF superfamily (e.g., 41BBL) and/or IL21 or IL7, are subsequently administered to the subject with cancer, optionally in the form of a pharmaceutical composition. The cancer can be, but is not limited to, melanoma, uveal (ocular) melanoma, cervical cancer, ovarian cancer, head and neck cancer, non-small cell lung cancer (NSCLC), bladder cancer, breast cancer, renal cell carcinoma, pancreatic cancer, prostate cancer, cancer of the central nervous system, gastrointestinal cancer (e.g., colorectal cancer).

[0098] T cell or TIL therapy to date often requires concomitant administration of high doses of IL2 simultaneously with and subsequent to administration of the T cells or TILs. But unlike conventional treatment with T cells or TILs, the present method does not require administration of IL2. Rather, the modified TILs or T cells by expressing mbIL15, provide a sufficient source of cytokine to stimulate proliferation and activity of the TILs and expansion on modified K562 cells in the absence of IL2 can allow for preferential expansion of the modified cells.

[0099] The method of treating cancer can further comprise isolating one or more T cells from a subject or TILs from a tumor as described herein and introducing into the one or more T cells or TILs a nucleic acid sequence that expresses a mbIL15 and optionally, a CAR or TCR. The T cells or TILs can be derived from a recipient subject (autologous source). The tumor from which the TILs are derived can be a primary tumor or a metastatic tumor. Thus, if TILs from a biopsy or portion of a primary tumor are cryopreserved, they can be thawed and used for treatment of a resulting metastatic tumor or different primary tumor at a later date. Alternatively, the T cells or TILs can be derived from a donor subject (allogeneic source), wherein the donor subject is not the recipient subject. TILs derived from the same tumor to be treated have the advantage of having neoantigens and heterogeneity that are comparable to that of the tumor. TILs derived from a different tumor of the same subject or from the tumor of the different donor can be selected for reactivity with cancer antigens that are present in the tumor of the recipient subject by methods known in the art such as tetramer staining of the TCR. If T cells or TILs are derived from a donor subject, wherein the donor subject is not the recipient subject, the method can further comprise selecting a donor subject that is a HLA match for the recipient subject, so as to reduce graft versus host responses.

[00100] T cells can be isolated from PBMCs, whereas TILs can be obtained from a tumor sample surgical resection, tissue biopsy, needle biopsy or other means as an initial step. The T cells or TILs are then transduced or modified as described herein and then expanded *in vitro* to provide a larger population of expanded cells for ACT.

[00101] Administration of the modified T cells or TILs expanded in the K562 cells can include an amount from about 1000 cells/injection to up to about 10 billion cells/injection, such as 2×10^{11} , 1×10^{11} , 1×10^{10} , 1×10^9 , 1×10^8 , 1×10^7 , 5×10^7 , 1×10^6 , 5×10^6 , 1×10^5 , 5×10^5 , 1×10^4 , 5×10^4 , 1×10^3 , or 5×10^3 , cells per injection, or any ranges between any two of the numbers, end points inclusive. Optionally, from 1×10^8 to 2×10^{11} cells are administered to the subject.

[00102] Modified T cells or TILs of the present disclosure can be administered by any suitable route. In some embodiments, the cells are administered by intravenous infusion, intra-arterial infusion, intraperitoneally, intrathecally, intralymphatically. Optionally, the modified T cells or TILs are administered locally, for example, directly into a tumor or blood vessel that supplies a tumor.

[00103] The modified T cells or TILs can be administered in a single dose, but in certain instances may be administered in multiple doses.

[00104] The method of treatment can further comprise lymphodepletion of the recipient subject prior to administration of the modified T cells or TILs. Investigations in humans and murine models of melanoma suggest that lymphodepletion depletes negative regulatory cells including regulatory T (T_{reg}) cells and peripheral myeloid-derived suppressor cells, which can suppress T cell proliferation. Thus, lymphodepletion aids in the proliferation of adoptively transferred T cells or TILs. Lymphodepleting conditioning regimens include, for example, pre-treatment of the recipient subject with full body irradiation and/or lymphodepleting agents before adoptive transfer of the T cells or TILs. This preconditioning allows the T cells or TILs to expand by eliminating T_{reg} cells and removing potential cytokine sinks by which normal cells compete with the newly infused T cells or TILs.

[00105] One example of an lymphodepleting agent is fludarabine (e.g., at a dose of 0.5 $\mu\text{g/ml}$ -10 $\mu\text{g/ml}$). In some embodiments, the fludarabine is administered at a concentration of 1 $\mu\text{g/ml}$ daily for 1-7 days before modified T cell or TIL administration. In some embodiments, the fludarabine is administered at a dosage of 10 mg/kg/day, 15 mg/kg/day, 20 mg/kg/day, 25 mg/kg/day, 30 mg/kg/day, 35 mg/kg/day, 40 mg/kg/day, or 45 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 2-7 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4-5 days at 35 mg/kg/day. In some embodiments, the fludarabine treatment is administered for 4-5 days at 25 mg/kg/day.

[00106] In some embodiments, cyclophosphamide is administered to provide mafosfamide, its active form, at a concentration of 0.5 $\mu\text{g/mL}$ -10 $\mu\text{g/mL}$. In some embodiments, cyclophosphamide is administered to provide mafosfamide at a concentration of 1 $\mu\text{g/mL}$ daily for 1-7 days before TIL administration. In some embodiments, the cyclophosphamide is administered at a dosage of 50 $\text{mg/m}^2/\text{day}$, 75 $\text{mg/m}^2/\text{day}$, 100 $\text{mg/m}^2/\text{day}$, 150 $\text{mg/m}^2/\text{day}$, 175 $\text{mg/m}^2/\text{day}$, 200 $\text{mg/m}^2/\text{day}$, 225 $\text{mg/m}^2/\text{day}$, 250 $\text{mg/m}^2/\text{day}$, 275 $\text{mg/m}^2/\text{day}$, or 300 $\text{mg/m}^2/\text{day}$. In some embodiments, the cyclophosphamide

is administered intravenously (i.v.). In some embodiments, the cyclophosphamide treatment is administered for 2-7 days at 35 mg/kg/day i.v. In some embodiments, the cyclophosphamide treatment is administered for 4-5 days at 250 mg/m²/day i.v. In some embodiments, the cyclophosphamide treatment is administered for 4 days at 250 mg/m²/day i.v.

[00107] In certain embodiments, lymphodepletion comprising administration of a combination of lymphodepleting agents, such as cyclophosphamide at 60 mg/kg for 2 days and fludarabine at 25 mg/m² for 5 days or cyclophosphamide 250mg/m²/day for 4 days and fludarabine at 25 mg/m² for 4 days.

[00108] If the IL15, CAR, or TCR expressed by the T cell or TIL is operably linked to a DRD, the method can further comprise administering to the recipient subject a second agent that binds to the DRD in an amount effective to increase the payload activity of the T cell or TIL. The ligand can be administered using a dosing regimen that provides a selected amount of payload activity to the subject. The ligand can be delivered to achieve continuous or intermittent payload activity in the subject. Determining the frequency and duration of dosing to the subject is determined by a person of skill in the art by considering, for example, a higher dose or longer duration of administration of the ligand when more activity of the payload is desired and reduces or eliminates the ligand administration when less activity is desired. The dose and duration of ligand administration and the resulting activity of the payload is also selected to avoid unacceptable side effects or toxicity in the subject. Thus, the subject is administered an effective amount of the ligand to achieve an effective amount of the payload. The term effective amount is defined as any amount necessary to produce a desired physiologic response. Effective amounts and schedules for administering the ligand may be determined empirically by one skilled in the art based on the amount of resulting payload, the activity of the payload, or based on one or more signs of the effect of the payload activity. The ranges for administration of the ligand range from zero to a saturating dose and the resulting payload activity ranges from a basal level to a maximum level, optionally with a sufficient dynamic range that allows for small changes in the amount of ligand to result in small changes in the amount of payload or payload activity. The dosage or frequency of the administration of the ligand and the resulting amount and activity of the payload should not be so large as to cause unacceptable adverse side effects and will vary with the patient's age, condition, sex, type of cancer being treated, the extent of the cancer,

and whether other therapeutic agents are included in the treatment regimen. Guidance can be found in the literature for appropriate dosages for given classes of ligands.

[00109] In some embodiments, the modified T cell or TILs with regulatable payloads (i.e., payloads operably linked to a DRD) can be administered in combination with one or more immune checkpoint regulators. Checkpoint inhibitors include antibodies that target PD-1 or inhibit the binding of PD-1 to PD-L1, including, but are not limited to, nivolumab (BMS-936558, Bristol-Myers Squibb; Opdivo®), pembrolizumab (Iambrolizumab, MK03475 or MK-3475, Merck; Keytruda®), humanized anti-PD-1 antibody JS001 (ShangHai JunShi), monoclonal anti-PD-1 antibody TSR-042 (Tesar, Inc.), Pidilizumab (anti-PD-1 mAb CT-011, Medivation), anti-PD-1 monoclonal Antibody BGB-A317 (BeiGene), and/or anti-PD-1 antibody SHR-1210 (ShangHai HengRui), human monoclonal antibody REGN2810 (Regeneron), human monoclonal antibody MDX-1106 (Bristol-Myers Squibb), and/or humanized anti-PD-1 IgG4 antibody PDR001 (Novartis).

[00110] The subject is optionally monitored for the outcome of the treatment. Thus, for example, the number of malignant cells in a sample, the circulating tumor DNA in a sample, or the size of a solid tumor upon imaging can be detected. If the desired end point is achieved (e.g., showing successful treatment of cancer), the ligand can be reduced or discontinued so as to reduce or eliminate the payload. Similarly, if the subject develops a cytokine storm, an allergic reaction, or other adverse effect from the payload, the ligand can be reduced or discontinued.

Definitions

[00111] The terms about and approximate, when used to refer to a measurable value such as an amount, concentration, dose, time, temperature, activity, level, number, frequency, percentage, dimension, size, weight, position, length and the like, is meant to account for variations due to experimental error, which could encompass variations of $\pm 15\%$, $\pm 10\%$, $\pm 5\%$, $\pm 1\%$, $\pm 0.5\%$, or even $\pm 0.1\%$ of the specified amount, concentration, dose, time, temperature, activity, level, number, frequency, percentage, dimension, size, weight, position, length and the like. All measurements or numbers are implicitly understood to be modified by the word about, even if the measurement or number is not explicitly modified by the word about. In instances in which the terms about and approximate are used in connection with the location or position of regions within a reference polypeptide, these terms encompass variations of $\pm$ up to 20 amino acid residues, $\pm$ up to 15 amino acid residues, $\pm$ up to 10

amino acid residues, $\pm$ up to 5 amino acid residues, $\pm$ up to 4 amino acid residues, $\pm$ up to 3 amino acid residues, $\pm$ up to 2 amino acid residues, or even $\pm$ 1 amino acid residue.

[00112] As used herein, operably linked means that, in the presence of a paired ligand, the DRD is linked to the payload directly or indirectly so as to alter a measurable characteristic of the payload (e.g., alters the level of activity of the payload as compared to the level of activity in the absence of the paired ligand). In some embodiments, the measured level of amount and/or activity of the payload increases in the presence of an effective amount of ligand as compared to the measured level of expression or activity in the absence of ligand. An effective amount the ligand means the amount of ligand needed to see an increase in the measure of the amount or activity of the payload. In some embodiments, the effective amount is not so great as to produce unacceptable toxicity or off-target effects. Optionally, the measurable characteristic is a therapeutic outcome, an amount of the payload in a sample, or a biological activity level of the payload (for which measuring the amount of payload can serve as a proxy).

[00113] Wherever the phrase linked or bound or the like is used, the phrase directly or indirectly is understood to follow unless explicitly stated otherwise or nonsensical in context. Thus, reference to a DRD linked to, bound to, or associated with a payload means in each case that a DRD is directly or indirectly linked to a payload.

[00114] As used herein the terms survival of TILs or of T cells and persistence of TILs or of T cells are used interchangeably. Survival is determined based on a persistent effect of the TILs or T cells.

[00115] As used herein, expansion is used to refer to a functional increase in cell number that occurs during a functional REP. A functional REP results in an expanded cell population that provides sufficient cell numbers for therapeutic use. An unsuccessful REP, on the other hand, would result in the absence of a functional fold increase in cell number. Unexpanded cells include pre-REP cells and those that have not undergone a functional expansion in REP as compared to an expanded cell population. A non-functional expansion includes expansion of 10% or less of an expanded cell population. For example, a TIL population that expanded 100-fold in a given time period can be compared to an unexpanded population that expanded only 10-fold or less. Thus, as used herein an expanded cell or expanded cell population refers to a cell or population of cells that has undergone a functional REP. An unexpanded cell or population of cells refers to a cell or population of cells pre-REP or subsequent to a REP that failed to result in functional expansion of the population of

cells. By way of example, certain modified TILs will expand on modified K562 feeder cells but the fold expansion on PBMCs will be less than 10% of the fold expansion on modified K562 feeder cells. Thus, the unexpanded TILs can be modified TILs pre-REP or modified TILs following REP on PBMCs.

[00116] The term identity as known in the art, refers to a relationship between two or more sequences, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between sequences, as determined by the number of matches between strings of two or more residues (amino acid or nucleic acid). Identity measures the percent of identical matches between two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., algorithms). Identity of related sequences can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988). Generally, variants of a particular polynucleotide or polypeptide of the disclosure will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, Thomas L. Madren, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.)

[00117] The term feeder cell as used herein refers to cells that support the expansion of TILs or T cells in culture, such as by secreting into the cell culture or presenting on the feeder cell membrane growth or survival factors. In some embodiments, feeder cells are growth arrested (i.e., replication incompetent).

[00118] As used herein, subject and patient are used synonymously and are not meant to be limited to human subjects or patients.

[00119] Treatment, as used herein refers to a reduction or delay in one or more signs or symptoms of the cancer. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Thus, efficacy of treatment or amelioration of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker, or any other measurable parameter appropriate for a given disease being treated or targeted for treating. In connection with the administration of compositions of the present disclosure, effective amount for treatment of cancer, indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as an improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of cancer.

[00120] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[00121] The details of one or more embodiments of the present disclosure are set forth in the description and accompanying drawings. It is to be understood that other embodiments may be utilized and structural or process changes made without departing from the scope of the disclosure. In other words, illustrative embodiments and aspects are described. But it will be appreciated that, in the development of any such actual embodiment, numerous implementation-specific decisions may be made to achieve the developer's specific goals, such as compliance with clinically relevant constraints, which may vary from one implementation to another. Moreover, it will be appreciated that such development effort might be complex and time-consuming but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

[00122] Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference in their entireties.

[00123] The examples below are intended to further illustrate certain aspects of the methods and compositions described herein, and are not intended to limit the scope of the claims.

Examples

Example 1: Isolation and Expansion of TIL From Patient Tumor Samples (pre-REP culture)

[00124] Melanoma and head and neck tumor samples were obtained from Cooperative Human Tissue Network. Tumor samples were cut into 1-3 mm fragments in Hanks' Balanced Salt Solution (HBSS) buffer and fragments were placed in multi-well plates at 1 fragment/well in 2 mL of TIL culture media (RPMI-1640 supplemented with GlutaMAX (Thermo Fisher), 1% Penicillin/Streptomycin, 1 mM Sodium Pyruvate, 1% HEPES, 50 μ M 2-Mercaptoethanol (Invitrogen) and 10% heat-inactivated human AB serum (Valley Bio)) containing 6000 IU/mL IL2 (Peprotech) and 0.1 mg/mL Normocin (InvivoGen). Half of the media was replaced with fresh media containing IL2 starting on day 5 and cells were split into multiple wells or flasks as they became confluent for a duration of 3 weeks. This culture process is referred to as pre-rapid expansion protocol (pre-REP). After pre-REP, TILs were aliquoted, frozen in cell freezing media (Bambanker, Bulldog Bio or Cryostor-10, STEMCELL Technologies) and stored long-term in liquid nitrogen.

[00125] In order to determine the change in frequency of T cells before and after pre-REP culture, a portion of tumor fragments were digested with collagenase and DNase I to generate single cell suspension prior to the pre-REP culture and compared to cells obtained after the pre-REP culture. Frequency of T cells were analyzed by flow cytometry using fluorochrome conjugated anti-CD45 and anti-CD3 antibodies. As shown in FIG. 1, nearly half of the cells ($44.29 \pm 21.67\%$) in the pre-culture tumor cell suspension were CD45+ and among these only approximately 40% ($\sim 39.85 \pm 23.69\%$) were CD3+ T cells. After 3 weeks of culture in the presence of IL2 (pre-REP), the majority of the cells were CD45+ ($90.35 \pm 7.28\%$), indicating an enrichment of hematopoietic cells, and CD3+ ($80.64 \pm 15.19\%$), indicating an enrichment of T cells.

[00126] TILs from other human tumor types, including melanoma tumors and malignant tumors from breast, lung, kidney, endometrium, liver, pancreas and ovary, were isolated in the same manner as described above.

Example 2: Generation of K562 Cells Expressing Membrane-Bound IL21 and 4-1BBL

Membrane-bound IL21 and 4-1BBL Vector Construct Assembly

[00127] The IL21-41BBL-001 insert comprises nucleic acid sequences encoding a leader sequence, membrane-bound IL21 (mbIL21), a P2A sequence and 4-1BBL. The mbIL21 nucleic acid sequence encodes, in order, an IL21 sequence, an IgG hinge, an IgG4 chain, a CD4 transmembrane domain and a Glycine-Serine (GS) linker (see Table 3). OT-IL21-41BBL-001, which comprises the IL21-41BBL-001 insert, was constructed in a pELNS vector (a third-generation self-inactivating lentiviral expression vector) using standard molecular biology techniques. Gene fragments (Gblocks) were inserted into the pELNS vector and placed under the control of the EF1a promoter using Gibson assembly (NEBuilder Hifi). The assembled plasmid was transformed into *E. coli* (NEB stable) for amplification and sequence was confirmed before proceeding with virus production.

Table 3: Components of a mbIL21-41BBL Construct

Description of Domain	NA Sequence	NA SEQ ID NO	AA Sequence	AA SEQ ID NO
GM-CSF Signal Peptide	ATGTGGCTGCAGTCTCTGCTCCTCTTGGGGACTGTCGCCTGTTCTATTTCA	32	MWLQSLLLLGTV ACSIS	33
IL21	CAAGGACAGGATCGACATATGATTCCGATGCGC CAACTGATAGATATAGTCGATCAACTCAAGAAT TATGTGAATGACTTGGTCCCTGAGTTTCTGCCGG CTCCAGAGGACGTTGAAACAAACTGTGAATGGT CAGCGTTTTTCATGTTTTCAAAGGCACAGCTCAA GTCCGCCAATACAGGCAATAACGAGCGGATTAT AAATGTCTCAATTAAAAAGCTCAAGCGCAAACC CCCTTCAACGAATGCTGGTCGCCGCCAGAAACA CAGGTTGACCTGTCCCTCCTGTGACTCATACGAG AAGAAACCTCCCAAGGAATTTCTCGAACGCTTT AAGTCACTCTTGCAAGAAGATGATTCATCAGCACT TGAGTAGCCGGACACATGGTTTCAGAGGATAGT	34	QGQDRHMIRMRL QLIDIVDQLKNYV NDLVPEFLPAPED VETNCEWSAFSC FQKAQLKSANTG NNERIINVSIIKKL KRKPPSTNAGRR QKHRLTCPSGDS YEKKPPKEFLERF KSLQKMIHQHL SSRTHGSEDS	35
IgG Hinge (S228P)	GAGTCTAAGTATGGCCCACCGTGTCCCCCCTGCCCA	36	ESKYGPPCPPCP	37

IgG4 Chain	GCACCTGAGTTCCTCGGAGGCCCTCTGTATTCC TGTTTCCCCCAAAGCCCAAGGATACTCTTATGAT CTCACGCACTCCGGAAGTAACCTGCGTGGTGGT GGATGTGAGTCAGGAAGACCCCGAAGTCCAGTT TAATTGGTACGTGGACGGGGTTGAGGTACATAA CGCCAAAACGAAACCTCGGGAGGAGCAATTCAA TTCCACTTACCGGGTTGTATCAGTCTGACTGTA CTGCATCAAGATTGGCTCAACGGGAAAGAGTAC AAGTGTAAGGTTAGTAATAAAGGGCTGCCGTCT AGTATTGAGAAAACGATCAGTAAGGCTAAAGGG CAGCCAAGAGAGCCACAAGTATATACCCTGCCA CCCTCTCAGGAGGAGATGACTAAAAACCAAGTG TCACTGACCTGCCTTGTTAAGGGTTTTTACCCAT CTGATATAGCAGTAGAGTGGAATCCAATGGAC AGCCAGAGAACAATTATAAGACTACACCTCCCG TCCTTGATAGTGACGGCTCCTTCTTCTTGATTCT CGACTTACAGTTGATAAGTCCCGCTGGCAGGAG GGTAATGTCTTTAGCTGCAGTGTAAATGCACGAA GCTCTTCATAATCACTACACAAAAATCATTGA GCCTGTCTCTGGGAAAG	38	APEFLGGPSVFLF PPKPKDTLMISRT PEVTCVVVDVSQ EDPEVQFNWYVD GVEVHNAKTKPR EEQFNSTYRVVS VLTVLHQDWLN GKEYKCKVSNKG LPSSIEKTISKAKG QPREPQVYTLPPS QEEMTKNQVSLT CLVKGFYPSDIAV EWESNGQPENNY KTPPVLDSDGSF FLYSRLTVDKSR WQEGNVFSCSVM HEALHNHYTQKS LSLSLGK	39
CD4 Trans- membrane	ATGGCCTTGATTGTGCTCGGCGGAGTTGCAGGCC TGCTCCTTTTTATTGGACTCGGAATATTTTTTC	40	MALIVLGGVAGL LLFIGLGIFF	41
Linker	GGATCTGGA	42	GSG	43
P2A	GCTACTAACTTCAGCCTGCTGAAGCAGGCTGGA GACGTGGAGGAGAACCCTGGACCT	44	ATNFSLLKQAGD VEENPGP	45
4-1BBL	ATGGAGTACGCTAGTGATGCGTCCTTGACCCC GAGGCGCCATGGCCACCGGCCCGCGAGCTCGA GCCTGTGAGTGCTGCCATGGGCTCTGGTCGCTG GGTTGCTCCTCCTTCTGCTTTTGGCCGCGGCTTGT GCAGTGTTTCTTGCTTGCCCGTGGGCAGTTAGCG GTGCTCGCGCATCTCCCGGAAGCGCGGCGAGTC CTCGACTCAGGGAAGGTCCGGAGCTGAGCCCAG ATGACCCCGCCGTTTGCTGGACCTCCGCCAAG GAATGTTTCGCTCAACTCGTTGCGCAAAACGTACT TCTTATAGACGGCCCTCTTAGTTGGTACAGTGAC CCAGGATTGGCTGGCGTTAGTTTGACAGGCGGA CTCAGTTACAAGGAGGATACTAAGGAACTGGTA GTCGCTAAGGCTGGGGTATACTACGTGTTCTTTC AACTCGAACTGAGAAGGGTGGTTGCGGGAGAAG GATCTGGAAGTGTATCTCTCGCCCTGCACCTCCA ACCCCTCAGAAGTGCCGCGGAGCGGCCGCCCT TGCCCTTACTGTGACCTGCCCCGGCTTCTTCA GAAGCGCGAAATAGTGCATTGCGCTTCCAGGGG CGCCTTTTGCACCTGAGCGCTGGACAGCGCCTCG GGGTCCACCTCCACACGGAAGCGCGGCGAGGC ACGCTTGCAACTCACACAAGGTGCGACGGTTC TCGGCTTGTTTAGGGTTACGCCTGAGATACCGGC TGGCCTCCCATCTCCAAGATCCGAG	46	MEYASDASLDPE APWPPAPRARAC RVLPPWALVAGLL LLLLLAAACAVF LACPWAVSGARA SPGSAASPRREG PELSPDDPAGLLD LRQGMFAQLVAQ NVI.IIDGPI.SWYS DPGLAGVSLTGG LSYKEDTKELVV AKAGVYYYVFFQL ELRRVVAGEGSG SVSLALHLQPLRS AAGAAALALTVD LPPASSEARNSAF GFQGRLLHLSAG QRLGVHLHTEAR ARHAWQLTQGA TVLGLFRVTPEIP AGLPSRSE	47
Linker	GGATCC	25	GS	24
Stop Codon	TAA	48	N/A	

IL21-41BBL insert	ATGTGGCTGCAGTCTCTGCTCCTCTTGGGGACTG TCGCTGTTCCTATTTTCAAGGACAGGATCGACA TATGATTCGGATGCGCCAAGTATAGATATAGTC GATCAACTCAAGAATTATGTGAATGACTTGGTCC CTGAGTTTCTGCCGGCTCCAGAGGACGTTGAAA CAAAGTGTGAATGGTCAGCGTTTTTCATGTTTTCA AAAGGCACAGCTCAAGTCCGCCAATACAGGCAA TAACGAGCGGATTATAAATGTCTCAATTAATAA GCTCAAGCGCAAACCCCTTCAACGAATGCTGG TCGCCGCCAGAAACACAGGTTGACCTGTCCCTCC TGTGACTCATAACGAGAAGAAACCTCCCAAGGAA TTTCTCGAACGCTTTAAGTCACTCTTGAGAGA TGATTCATCAGCACTTGAGTAGCCGGACACATG GTTTCAAGGATAGTGAGTCTAAGTATGGCCAC CGTGTCCCCCTGCCAGCACCTGAGTTCTCGG AGGCCCTCTGTATTCCTGTTTCCCCCAAAGCCC AAGGATACTCTTATGATCTCACGCACTCCGGAA GTAACCTGCGTGGTGGTGGATGTGAGTCAGGAA GACCCCGAAGTCCAGTTTAATTGGTACGTGGAC GGGGTTGAGGTACATAACGCCAAAACGAAACCT CGGGAGGAGCAATTCAATTCCACTTACCGGGTT GTATCAGTCCTGACTGTACTGCATCAAGATTGGC TCAACGGGAAAGAGTACAAGTGTAAGGTTAGTA ATAAAGGGCTGCCGTCTAGTATTGAGAAAACGA TCAGTAAGGCTAAAGGGCAGCCAAGAGAGCCAC AAGTATATACCCTGCCACCCTCTCAGGAGGAGA TGACTAAAAACCAAGTGTCACTGACCTGCCTTGT TAAGGGTTTTTACCCATCTGATATAGCAGTAGAG TGGAATCCCAATGGACAGCCAGAGAACAATTAT AAGACTACACCTCCCGTCTTGATAGTGACGGCT CCTTCTTCTTGTATTCTCGACTTACAGTTGATAA GTCCCGCTGGCAGGAGGGTAATGTCTTTAGCTGC AGTGTAATGCACGAAGCTCTTCATAATCACTACA CACAAAAATCATTGAGCCTGTCTTGGAAGA TGGCCTTGATTGTGCTCGGCGGAGTTGCAGGCCT GCTCCTTTTTATTGGAAGTCTGGAATATTTTTCGGA TCTGGA_{gctactaactcagcctgctgaagcaggctggagacgtggagga} _{gaacctggacct}ATGGAGTACGCTAGTGATGCGTCCT TGGACCCCGAGGCGCCATGGCCACCGGCCCGC GAGCTCGAGCCTGTCGAGTGCTGCCATGGGCTCT GGTCGCTGGGTTGCTCCTCCTTCTGCTTTTGGCC GCGGCTTGTGCAGTGTTTCTTGCTTGCCCGTGGG CAGTTAGCGGTGCTCGCGCATCTCCCGGAAGCG CGGCGAGTCTCGACTCAGGGAAGGTCCGGAGC TGAGCCCAGATGACCCCGCCGGTTTGTGACCT CCGCCAAGGAATGTTTCGCTCAACTCGTTGCGCA AAACGTACTTCTTATAGACGGCCCTCTTAGTTGG TACAGTGACCCAGGATTGGCTGGCGTTAGTTGA CAGGCGGACTCAGTTACAAGGAGGATACTAAGG AACTGGTAGTCGCTAAGGCTGGGGTATACTACG TGTTCTTTCAACTCGAACTGAGAAGGGTGGTTGC GGGAGAAGGATCTGGAAGTGTATCTCGCCCT GCACCTCCAACCCCTCAGAAGTGCCGCCGGAGC GGCCGCCCTTGCCCTTACTGTGACCTGCCCCCG	49	MWLQSLLLLGTV ACISQGDHRMI RMRQLIDIVDQL KNYVNDLVPEFL PAPEDVETNCEW SAFSCFQKAQLKS ANTGNNERIINVS IKKLKRKPPSTNA GRRQKHRLTCPS CDSYEKKPPKEFL ERFKSLLQKMIH QHLSSRTHGSEDS ESKYGPPCPPCPA PEFLGGPSVFLFP PKPKDTLMIS RTP EVTCVVVDVSQE DPEVQFNWYVD GVEVHNAKTKPR EEQFNSTYRVVS VLTVLHQDWLN GKEYKCKVSNKG LPSSIEKTISKAKG QPREPQVYTLPPS QEEMTKNQVSLT CLVKGFYPSDIIV EWESNGQPENNY KTPPVLDSDGSF FLYSRLTVDKSR WQEGNVFSCSVM HEALHNHYTQKS LSLSLGKMALIVL GGVAGLLLFILGL IFFGSGATNFSLL KQAGDVEENPGP MEYASDASLDPE APWPPAPRARAC RVLPWALVAGLL LLLLLAAACAVF LACPWAVSGARA SPGSAASPRREG PELSPDDPAGLLD LRQGMFAQLVAQ NVLLIDGPLSWYS DPGLAGVSLTGG LSYKEDTKELVV AKAGVYYVFFQL ELRRVVAGEGSG SVSLALHLQPLRS AAGAAALALTVD LPPASSEARNSAF GFQGRLLHLSAG QRLGVHLHTEAR ARHAWQLTQGA	50
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	GCTTCTTCAGAAGCGCGAAATAGTGCATTTCGGCT TCCAGGGGCGCCTTTTGCACCTTGAGCGCTGGACA GCGCCTCGGGGTCCACCTCCACACGGAAGCGCG GGCGAGGCACGCTTGGCAACTCACACAAGGTGC GACGGTTCTCGGCTTGTTTAGGGTTACGCCTGAG ATACCGGCTGGCCTCCCATCTCCAAGATCCGAG GGATCC _{taa}		TVLGLFRVTPEIP AGLPSRSEGS*	
OT-IL21- 41BBL-001	gcgcgctcactggccgctgtttacaacgtcgtgactgggaaaaccctggcgta cccaactaatcgcttgagcacatcccccttgcagctggcgtaatagcga agaggcccgaccgatcgccctcccaacagtgcgcagcctgaatggcgaa gggacgcgcctgtagcggcgcaataagcgcggcggtgtgtgtgtacgcg cagcgtgaccgctacactgcccagcgccctagcggcgctccttctccttcc cttcttctcgccacgttcgcccgttccccgtcaagctctaaatcgggggctc ccttagggttccgatttagtgccttacggcacctcgaccccaaaaactgattag ggtagtggttcacgtagtgggccatcgccctgatagacgggttttgcctttgac gttgagtgccacgttcttaatagtggactctgttccaaactggaacaactcaa ccctatctcggtctattctttgattataagggttttgcgatttcggcctattggt aaaaatgagctgatttaacaaaaattaacgcgaatttaacaaaatattaacgt tacaatttaggtggcacttttcggggaaatgtgcgcggaacccctatttattt tctaaatacattcaaatatgtatccgctcatgagacaataaccctgataatgcttc aataatattgaaaagggaagagtatgagtattcaacatttcggtgcgccctattc cctttttgcggcattttgcctcctgttttgcaccagaaacgctgggtaaagt aaaagatgctgaagatcagttgggtgcacgagtggttacatgaactggatct caacagcggtaagatcctgagagtttgcggcggaagacgtttccaatgatg agcacctttaaagttctgctatgtggcgcggtatfatcccgtaftgacgcccggca agagcaactcggtcgcccgcatacactatttcagaatgacttggttgagtactca ccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagt gctgccataaccatgagtataacactgcggccaacttacttctgacaacgatcg gaggaccgaaggagctaaccgctttttgcacaactgggggatcatgtaactc gccttgatcgttggaaccggagctgaatgaagccataccaaacgacgagcgt gacaccacgatgcctgtagcaatggcaacaacgttcgcaactattaactggc gaactacttactctagcttcccggcaacaattaatagactggatggaggcgata aagttgcaggaccacttctgcgctcgccctccggctggtgtgttattgtgat aatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggcc agatggtaagccctccgtatcgtatctacacgacggggagtcaggcaac tatggatgaacgaatagacagatcgctgagatagtgccctactgattaagcat tggttaactgtcagaccaagttactcatatatacttttagattgatttaaaacttcatttt aatttaaaagatctaggtgaagatccttttgataatctcatgaccaaataccctta acgtgagtttctgctccactgagcgtcagacccgtagaaaaagatcaaaaggatct tcttagatccttttttctgcgctaatctgctgcttgcaacaaaaaaaccaccg ctaccagcgggtgtgttgcggatcaagagctaccaactcttttccgaaggt aactggcttcagcagagcgcagataccaaatactgttctctagtgtagccgtagt	51	N/A	

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[00128] Table 3 presents the nucleic acid and amino acid sequences for domains of a mbIL21-41BBL construct disclosed herein. OT-IL12-(241-262) and OT-CD19-IL12-(297-316, 319-332) plasmids were each constructed in a pELNS vector (a third-generation self-inactivating lentiviral expression vector) using standard molecular biology techniques. Gene fragments (Gblocks or strings DNA) encoding IL12, Glycine-serine linkers, various hinges, transmembrane domains and cytoplasmic tails were purchased from Integrated DNA Technologies or Thermo-fisher scientific. The gene fragments were inserted into the pELNS vector and placed under the control of the EF1a promoter using Gibson assembly (NEBuilder Hifi). The assembled plasmid was transformed into *E. coli* (NEB stable) for amplification and sequence confirmed before proceeding with virus production.

OTLV-IL21-41BBL-001 lentivirus production

[00129] On the day of transfection, HEK293T cells were seeded in collagen-coated tissue culture flasks with 15×10^6 cells/flask in a total volume of 20 mL growth media (Dulbecco's Modified Eagle Medium (DMEM), 5% fetal bovine serum (FBS), and 1% penicillin/streptomycin). One hour before transfection, the growth media was replaced with warmed SFM4Transfx-293. Cells were transfected using Lipofectamine 3000 transfection reagent and P3000 enhancer reagent in Opti-MEM media with OT-IL21-41BBL-001 and packaging plasmids pRSV.Rev, pMDLg/pRRE, and pMD2.G (Addgene #122590). Media was replaced 6-8 hours (hr) post-transfection with SFM4Transfx-293. Supernatants containing OTLV-IL21-41BBL-001 were harvested 24 hr post-transfection, fresh media was added, and supernatants were harvested again at 48 hr post-transfection. Viral supernatants were filtered to remove debris and concentrated by ultracentrifugation at 25,000g for 2 hr at 4°C. The OTLV-IL21-41BBL-001 lentivirus was resuspended, aliquoted, and stored at -80°C.

Transduction of K562 cells with OTLV-IL21-41BBL-001 lentivirus

[00130] K562 cells were cultured in growth media containing RPMI-1640 with 2 mM L-Glutamine and 10% FBS (complete RPMI, Thermo Fisher). On the day of transfection,

K562 cells were seeded in multi-well plates at 1.5×10^5 cells/well in 500 μ L K562 cell growth media. The cells were transduced with OTLV-IL21-41BBL-001 lentivirus and then centrifuged at 800g for one hour at 32°C. Cells were incubated for 24-48 hours and then assessed for viability and expression of IL21 and 4-1BBL by flow cytometry using antibodies eFluor 780 (Thermo Fisher, 1:1000), 4-1BBL phycoerythrin (1:50), and IL21 allophycocyanin (1:50). The transduced K562 cells were expanded in complete RPMI for 17 days, and subsequently aliquoted, frozen using cell freezing media (Bambanker, Bulldog Bio), and stored in liquid nitrogen long-term. These transduced K562 will be referred to as K562-IL21-41BBL in this document.

[00131] The K562-IL21-41BBL cells were irradiated or treated with mitomycin C prior to their use as feeder cells in the TIL REP process. For irradiation, K562-IL21-41BBL cells were taken from fresh cell culture, centrifuged and resuspended in complete RPMI at $5-20 \times 10^6$ cells/mL. Resuspended cells were exposed to 50-200Gy in an X-ray irradiator, following which cells were washed and resuspended at 3×10^6 cells/mL for immediate use in the REP process. For mitomycin C treatment, the cells were thawed, centrifuged and resuspended in TIL media at 5×10^6 cells/mL. 10 μ g/mL Mitomycin-C was added to the cells and the cells were incubated for 30 minutes at 37°C. The cells were then washed three times with 50 mL TIL media and resuspended at 3×10^6 cells/mL for immediate use in the REP process.

Example 3. Transduction of TIL with Lentiviral Vectors

IL15 Vector Construct Assembly

[00132] OT-IL15-292 and OT-IL15-293 (sequences below) were each constructed in a pELNS vector (a third-generation self-inactivating lentiviral expression vector) using standard molecular biology techniques. Gene fragments (Gblocks) encoding codon-optimized IL15, GS linker, B7-1 hinge, transmembrane domain and cytoplasmic tails were purchased from Integrated DNA Technologies, Inc. (IDT, Coralville, Iowa). The gene fragments were inserted into the pELNS vector and placed under the control of the EF1a promoter using Gibson assembly (NEBuilder Hifi). The assembled plasmid was transformed into *E. coli* (NEB stable) for amplification and sequence confirmed before proceeding with virus production.

[00133] Table 1 and Table 2 (provided above) present the nucleic acid and amino acid sequences for components of a constitutive mbIL15 construct (OT-IL15-292) and an ACZ-

regulated mbIL15 construct (OT-IL15-293) disclosed herein. Construct OT-IL15-293 comprises a destabilizing domain labeled as CA2 (M1del, L156H) in Table 1.

[00134] Table 2 (provided above) also presents the nucleic acid and amino acid sequences of the constitutive IL15 (IL15-292) and ACZ-regulated IL15 (IL15-293) constructs disclosed herein.

BaEV-pseudotyped lentivirus production

[00135] HEK293T cells were seeded on collagen coated tissue culture plates until 70% confluent. Cells were transfected with pELNS transfer vector carrying constitutive (IL15-292) or regulated (IL15-293) IL15 constructs, as well as packaging plasmids pRSV.Rev (Addgene #12253), pMDLg/pRRE (Addgene #12251) and OT-BaEVg-002 (SEQ ID NO: XX) using Lipofectamine 3000 transfection reagent and P3000 enhancer reagent (Thermo Fisher) in Opti-MEM media (Thermo Fisher). Media was replaced 6-8 hours (hr) post-transfection with serum-free media (SFM4Transfx-293, Cytiva). Supernatants containing virus were harvested 24 hr post-transfection, fresh media was added, and supernatants were harvested again at 48 hr post-transfection. Viral supernatants were filtered to remove debris and concentrated by low-speed ultracentrifugation. Virus were resuspended, aliquoted and stored at -80oC.

Rapid Expansion Protocol (REP) and Transduction of TILs with BaEV-Pseudotyped Lentiviral Vectors

[00136] TILs generated from a head and neck tumor sample prepared as described in Example 1 were engineered after 3 weeks in the pre-REP culture. TILs were thawed and rested overnight in TIL media with 6000 IU/mL human IL2. TILs were then activated for 24 hr in 24-well plates with anti-CD3/CD28 beads (Dynabeads, Thermo Fisher) at 3:1 bead to TIL ratio or with plate-bound OKT3 at 3 µg/mL (Ultra-LEAF purified anti-human CD3 antibody, Biolegend) and 6000 IU/mL human IL2. RetroNectin (30 µg/mL) was used to coat 96-well non-coated cell culture plates overnight at 4°C. The following day, RetroNectin was removed, the plates were blocked with 2% bovine serum albumin (BSA) in PBS, and the plates were then washed with PBS. BaEV-pseudotyped lentivirus supernatants, prepared as described above, were diluted in TIL media and added in a total volume of 100-200 µL per well for an MOI of 1-4 TU/cell. The plates containing viral vector were centrifuged at 1400g for 2 hr at 32oC, and the supernatant was then removed. After supernatant removal, 1.5×10^5

activated TILs were transferred per well with 0 - 6000 IU/mL IL2 and incubated at 37°C overnight. Cells were processed similarly without virus addition and used as negative control (“unengineered”). 24 hours after transduction, TILs were transferred into a 6M GREX well plate (Wilson Wolf) in a total of 16-40 mL TIL media (RPMI-1640 supplemented with GlutaMAX (Thermo Fisher), 1% Penicillin/Streptomycin, 1 mM Sodium Pyruvate, 1% HEPES, 50 µM 2-Mercaptoethanol (Invitrogen) and 10% heat-inactivated human AB serum (Valley Bio)). Irradiated or Mitomycin-C treated K562 feeder cells transduced with 41BBL and mbIL21 as described in Example 2 were added to the culture at a ratio of 2:1 or 5:1 K562 to TIL. TILs transduced with the regulated mbIL15 construct received 25 µM Acetazolamide (SelleckChem) and untransduced TILs received 6000 IU/mL IL2. The cells were grown for 14 days in the GREX plates for the “rapid expansion protocol” or REP, and media was added or replaced as necessary. During the expansion, each GREX well was resuspended and mixed thoroughly, and an aliquot was taken for cell counting (Cellaca Cell Counter, Nexcelom) and flow cytometry staining. Samples were stained using antibodies CD3-BUV395 (BD), CD56-BV711 (Biolegend), CD4-BV605 (Biolegend), CD8-Alexa Fluor 700 (Biolegend), IL15-DyL650 (LakePharma, conjugated in-house), IL15RaFc-Biotin (ACRO Biosystems) with secondary Streptavidin-BV421 (Biolegend), and fixable viability dye eFluor 780 (Thermo Fisher). Samples were run on the BD Fortessa flow cytometer and analysis conducted using Flow Jo V10.7.1. The transduction efficiency was determined by percent of cells staining double positive for IL15-DyL650 and IL15RaFc-Biotin within the population of live, CD3 positive cells (FIG. 2).

[00137] TILs transduced with lentivirus comprising nucleic acid sequences encoding mbIL15 as described herein may be referred to in subsequent examples as “mbIL15 TILs.” TILs transduced with lentivirus comprising nucleic acid sequences encoding regulated mbIL15, such as OT-IL15-293, may also be referred to in subsequent examples as “regulated mbIL15 TILs.” TILs transduced with lentivirus comprising nucleic acid sequences encoding constitutive mbIL15, such as OT-IL15-292, may also be referred to in subsequent examples as “constitutive mbIL15 TILs.”

Example 4. TIL expansion in Rapid Expansion Protocol

[00138] TILs and feeder cells were generated as described in Examples 1-3 above. Briefly, after 3 weeks in the pre-REP culture, cryopreserved TILs were thawed and rested overnight with 6000 IU/mL human IL2. TILs were then activated with anti-CD3/CD28

Dynabeads or on OKT3-coated multi-well plates for 24 hours, after which point they were transduced with constitutive mbIL15 (OT-IL15-292) or GFP (OT-EGFP-001) lentiviral vectors or unmodified as an unengineered condition. 24 hours after transduction, TILs were expanded with K562-IL21-41BBL feeder cells (2:1 ratio of feeder cells:TILs) in GREX 6M well plates (Wilson Wolf) with 6000 IU/mL I-2 added to unengineered TILs as well as experimental “+IL2” conditions. The cells were grown for 12 days in the GREX plates for the “rapid expansion protocol” or REP, and media was added or replaced as necessary. On days 5, 8, and 12 post-transduction, each GREX well was resuspended. An aliquot was taken for flow cytometry staining to quantify the number of IL15+ or GFP+ cells as described in Example 3 using antibodies CD3-BUV395 (BD), CD56-BV711 (Biolegend), CD4-BV605 (Biolegend), CD8-Alexa Fluor 700 (Biolegend), IL15-DyL650 (LakePharma, conjugated in-house), IL15RaFc-Biotin (ACRO Biosystems) with secondary Streptavidin-BV421 (Biolegend), and fixable viability dye eFluor 780 (Thermo Fisher). GFP-expressing TILs require exogenous IL2 for expansion in REP, while constitutive mb-IL15-expressing TILs expand in the absence of IL2 (FIG. 3A).

Example 5. Expansion and survival in an antigen-independent setting

[00139] Next, post-REP TILs for assessed for their ability to persist or expand in the context of an in vitro antigen-independent survival assay. After 12 days of REP expansion, mbIL15 transduced cells that were expanded with no cytokine and GFP cells that were expanded with 6000 IU/mL IL2 were de-beaded, washed, and rested overnight with no cytokine. The next day, TILs were plated in a 48-well plate at 5×10^5 cells/well in TIL media with or without added IL2 (6000 IU/mL, Peprotech). Cells were split or media was added every two days for a total duration of 10 days. An aliquot was also taken for flow cytometry staining every two days and the number of IL15+ or GFP+ cells was quantified as described in Example 3. Constitutive mbIL15 TILs expanded during the 14-day survival assay either with or without exogenous IL2, while GFP TILs required IL2 for expansion (FIG. 3B).

[00140] In a new study that included regulated mbIL15 expressing TILs, TILs and feeder cells were generated as described in Examples 1-3 above. Briefly, after 3 weeks in the pre-REP culture, cryopreserved TILs were thawed and rested overnight with 6000 IU/mL human IL2. TILs were then activated with anti-CD3/CD28 Dynabeads or on OKT3-coated multi-well plates for 24 hours, after which point they were transduced with constitutive mbIL15 (OT-IL15-292) or regulated mbIL15 (OT-IL15-293) lentiviral vectors or

unengineered. 24 hours after transduction, TILs were expanded with K562-IL21-41BBL feeder cells (5:1 ratio of feeder cells:TILs) in GREX 6M well plates (Wilson Wolf) with 6000 IU/mL IL2 added to UT TILs, and 25 μ M acetazolamide (SelleckChem) added to regulated mbIL15 TILs. After 14 days of expansion, TILs were isolated and plated in a multi-well plate at 5×10^5 cells/well in TIL media with or without added IL2 (200IU/mL, Peprotech) or acetazolamide (25 μ M, SelleckChem). Entire wells were harvested for analysis of cell expansion by cell count (Celleca Cell Counter, Nexelom) and phenotype by flow cytometry (BD Fortessa) and fresh cytokine/ligand was added every 3 days. As demonstrated in FIG. 4, over the course of the 15 days of this assay, unengineered TILs did not expand without any exogenous cytokines (0.07 ± 0.03 -fold expansion), but with exogenous IL2 (200 IU/mL) were able to expand greater than twenty-fold (27.8 ± 0.25 -fold expansion). In contrast, modified TILs expanded significantly without the addition of any exogenous cytokines; after 15 days constitutive mbIL15 TILs expanded eight-fold (8.28 ± 1.9 -fold expansion), and regulated mbIL15 TILs given 25 μ M acetazolamide expanded seventeen-fold (17.3 ± 0.82 -fold expansion). Without the addition of acetazolamide, regulated mbIL15 TILs expanded four-fold lower than with ligand (4.52 ± 0.48 -fold expansion), highlighting the role of acetazolamide in regulating survival of regulated mbIL15 TILs.

Example 6. Expansion and survival in an antigen-dependent setting

[00141] TILs and feeder cells were generated as described in Examples 1-3 above. Briefly, after 3 weeks in the pre-REP culture, cryopreserved TILs were thawed and rested overnight with 6000 IU/mL human IL2. TILs were then activated with anti-CD3/CD28 Dynabeads or on OKT3-coated multi-well plates for 24 hours, after which point they were transduced with regulated mbIL15 (OT-IL15-293) lentiviral vectors or unengineered. Twenty-four hours after transduction, TILs were expanded with K562-IL21-41BBL feeder cells (5:1 ratio of feeder cells:TILs) in GREX 6M well plates (Wilson Wolf) with 6000 IU/mL IL2 added to UT TILs, and 25 μ M acetazolamide (SelleckChem) added to regulated mbIL15 TILs. After 14 days of expansion, TILs were cryopreserved in Bambanker freezing medium (Bulldog Bio). At a later time, cryopreserved TILs were thawed and rested overnight in TIL media with 200IU/mL IL2 (unengineered TILs) or TIL media with 25 μ M acetazolamide (regulated mbIL15 TILs). Following overnight rest, TILs were plated in a multi-well plate at 1:1 ratio with mitomycin C-treated melanoma cells in a TIL:tumor co-culture assay in TIL media with or without added IL2 (200IU/mL, Peprotech) or

acetazolamide (25 μ M, SelleckChem), and the assay was sustained for 27 total days. A vehicle-only control was included for acetazolamide, with the identical volume of DMSO added to vehicle control groups. Melanoma cells were from the A375 cell line (ATCC), which was modified with a puromycin-dependent luciferase vector, and were treated with 10 μ g/mL mitomycin C as described above (Example 3) to prevent proliferation of these tumor cells. Every 3 days, wells of this co-culture assay were mixed and an aliquot was isolated for analysis of cell expansion by cell count (Celleca Cell Counter, Nexelom) and phenotype by flow cytometry (BD Fortessa). Fresh mitomycin C-treated A375 melanoma cells as well as fresh cytokine/ligand in TIL media was added every 3 days. As demonstrated in FIG. 5, regulated mbIL15 TILs regulated with acetazolamide establish stable expansion kinetics, and even in this antigen-dependent setting, where the chronic stimulation should rapidly exhaust TILs and decrease cell counts, transduced TILs persisted. Over the assay, unengineered TILs did not expand without any exogenous cytokines (0.46 ± 0.02 -fold expansion from day 1 to day 27), but with exogenous IL2 (200 IU/mL) were able to expand greater than twenty five-fold (25.4 ± 4.06 -fold expansion from day 1 to day 27). In contrast, modified TILs expanded without the addition of any exogenous cytokines and notably regulated mbIL15 TILs given 25 μ M acetazolamide expanded twelve-fold (12.2 ± 0.10 -fold expansion from day 1 to day 27). Without the addition of acetazolamide (with vehicle control only), regulated mbIL15 TILs expanded four-fold lower than with ligand (2.68 ± 0.42 -fold expansion from day 1 to day 27), highlighting the role of acetazolamide in regulating survival of regulated mbIL15 TILs.

Example 7. Tumor reactivity of fresh post-REP TILs

[00142] TILs from two melanoma donors were generated as described in Examples 1-3. Briefly, after 3 weeks in the pre-REP culture, cryopreserved TILs were thawed and rested overnight with 6000 IU/mL human IL2. TILs were then activated with anti-CD3/CD28 Dynabeads or on OKT3-coated multi-well plates for 24 hours, after which point they were transduced with regulated mbIL15 (OT-IL15-293) lentiviral vectors or unengineered. 24 hours after transduction, TILs were expanded with K562-IL21-41BBL feeder cells (5:1 ratio of feeder cells:TILs) in GREX 6M well plates (Wilson Wolf) with 6000 IU/mL IL2 added to unengineered TILs, and 25 μ M acetazolamide (SelleckChem) added to regulated mbIL15 TILs. After 14 days of expansion, TILs were harvested, de-beaded, and rested overnight with and without IL2 and acetazolamide. Melanoma cell line expressing luciferase, A375-FLuc-Puro (ATCC) was resuspended in TIL media at 5×10^6 cells/mL. 10 μ g/mL Mitomycin-C

was added to the cells, which were then incubated for 30 minutes at 37°C. The cells were then washed three times with 50 mL TIL media. 1×10^5 A375 cells per well were added to a 96-well flat bottom tissue-culture treated plate. In some wells, 80 µg/mL HLA-ABC (Biolegend) blocking antibody was added to block MHC class I on the target cells. TILs that were rested overnight were added at a 1:1 or 3:1 ratio of TIL:A375 for a total volume of 200 µL per well. At a 48-hour time point, supernatant was saved from each well and the concentration of IFN γ was assayed by MSD. Lysis of the tumor cells was analyzed using CellTiterGlo Luminescent Cell Viability Assay (Promega), following manufacturer's protocol. Percent lysis was calculated as luminescence in the co-culture well minus background fluorescence divided by luminescence in A375-only control wells minus background fluorescence. Both untransduced TILs cultured with IL2 and regulated mbIL15 TILs expanded in REP in the absence of IL2 produce increased IFN γ in co-culture with the A375 melanoma line compared to TILs alone (FIG. 6A). Additionally, there was specific lysis of the tumor cells in co-culture conditions measured by decreased luminescence of the target cell line (FIG. 6B). Both percent specific lysis and IFN γ production was decreased in co-culture conditions with MHC class I blocking antibody, indicating that the cytotoxicity of the TILs against this tumor cell line is MHC class I dependent. This result is repeated in two melanoma donors.

Example 8. mbIL15 TILs persist long-term *in vivo* without IL2

[00143] TILs from one donor and feeder cells were generated as described in Examples 1-3 above. Briefly, after 3 weeks in the pre-REP culture, cryopreserved TILs were thawed and rested overnight with 6000 IU/mL human IL2. TILs were then activated with anti-CD3/CD28 Dynabeads for 24 hours, after which point they were transduced with constitutive mbIL15 (OT-IL15-292) or regulated mbIL15 (OT-IL15-293) lentiviral vectors or unengineered. 24 hours after transduction, TILs were expanded with K562-IL21-41BBL feeder cells (5:1 ratio of feeder cells:TILs) in GREX 6M well plates (Wilson Wolf) with 6000 IU/mL IL2 added to unengineered TILs, and 25 µM acetazolamide (SelleckChem) added to regulated mbIL15 TILs. After 14 days of expansion, TILs were harvested, de-beaded, and prepared for adoptive cell transfer. Unengineered TILs expanded 612-fold, constitutive mbIL15 TILs expanded 1080-fold, and regulated mbIL15 TILs expanded 450-fold (FIG. 7A).

[00144] NSG (NOD.Cg-Prkdcscid1l2rgtm1Wjl/SzJ) mice were purchased from Jackson Laboratories. Six- to eight-week-old female mice were systemically infused with 10

$\times 10^6$ TILs/mouse, with or without exogenous IL2 (Proleukin), or clinical grade acetazolamide or vehicle, as described in Table 4.

Table 4: *In Vivo* Group Dosing

Experimental Group	Cells	Exogenous Treatment	N
Unengineered	10×10^6 Untransduced TILs/mouse		10
Unengineered + IL2	10×10^6 Untransduced TILs/mouse	6×10^5 IU Proleukin/mouse, dosed IP QD for the first 4 days on study	10
Constitutive mbIL15	10×10^6 constitutive mbIL15 transduced TILs/mouse		10
Regulated mbIL15 + ACZ	10×10^6 regulated mbIL15 transduced TILs/mouse	200mg/kg PO QD acetazolamide, dosed daily	10
Regulated mbIL15 + vehicle	10×10^6 regulated mbIL15 transduced TILs/mouse	200mg/kg PO QD vehicle, dosed daily	10

[00145] TILs were assessed for IL15 expression on the day of adoptive cell therapy, and constitutive mbIL15 transduced TILs exhibited slightly higher levels of mbIL15 transduction (30.2 ± 0.46 % IL15+IL15RaFc+) than regulated mbIL15 transduced TILs (23.6 ± 1.1 % IL15+IL15RaFc+), but both transduced populations were acceptable for adoptive cell transfer (FIG. 7B). IL15 expression or transduction efficiency was assessed by flow cytometry; cells were incubated with Fc Block, and stained first with IL15 conjugated to DyL650 (Lake Pharma, conjugated in-house) and biotinylated IL15RaFc (ACROBiosystems). After incubating in the dark at room temperature for 25 minutes, cells were washed in FACS buffer, centrifuged, and resuspended in FACS buffer containing streptavidin conjugated to BV421 (Biolegend). After incubating in the dark at 4°C for 20 minutes, cells were washed in FACS buffer, centrifuged, resuspended in FACS buffer, and samples were run on BD Fortessa flow cytometer. Analysis occurred with FlowJo V10.7.1.

[00146] On days 7, 14, 21, 32, 39, 46, and 53 following adoptive cell therapy, 75 μ L of systemic blood was isolated via submandibular vein collection in EDTA-containing tubes and processed for enumeration of TILs. Blood samples received 1-3 mL of ACK lysis buffer (Gibco) and were incubated for 10-20 minutes to lyse red blood cells (RBCs). After RBC lysis, samples were filtered through a 70 μ m cell strainer, centrifuged, and resuspend in FACS buffer. An aliquot of each sample was isolated for analysis of cell count (Celleca Cell Counter, Nexelom) and the remainder was used for phenotype assessment by flow cytometry (BD Fortessa). For phenotypic assessment, blood samples were stained with antibodies specific for CD3 (BD), mouse CD45, CD25 (BD), FoxP3, CD4, CD8, IL15 (Lake Pharma, conjugated in-house), KLRG1, CD127, CD45RA, CD45RO, CD95, CD69, CCR7, CD56, and biotinylated IL15RaFc (ACROBiosystems). Antibodies were conjugated to FITC, PE, PE-Cy5, PE-Cy7, PerCP-Cy5.5, DyL650, APC-Cy7, BUV395, BUV737, BV421, BV510, BV605, BV711, or BV786 (Anti-human antibodies, all Biolegend, unless otherwise identified). In addition, a viability dye (e780 fixable viability dye, Invitrogen) was included for all samples. Samples were run on the BD Fortessa flow cytometer and analysis conducted using Flow Jo V10.7.1. To enumerate TILs throughout the study, TILs were gated as live cells, followed by lymphocytes, followed by human CD3⁺ and mouse CD45⁻ cells. As seen in FIG. 8A, unengineered TILs rapidly declined *in vivo*, reaching undetectable levels by day 53 post-infusion. Unengineered TILs receiving exogenous IL2 fared better, although persistence was low by day 53 post-infusion, where quantified TILs were at 0.64 ± 0.17 %. In contrast, by day 53-post infusion it was clear that transduced TILs remained at detectable levels systemically, with constitutive mbIL15 TILs at 5.73 ± 1.2 %. And regulated mbIL15 TILs + ACZ at 10.2 ± 2.0 %. The *in vivo* regulation effect of acetazolamide was clear, as by day 53 post-infusion regulated mbIL15 TILs + vehicle were nearly undetectable, at 2.94 ± 0.36 %.

[00147] On days 14 and 53 following adoptive cell therapy, a cohort of 5 animals per experimental group were sacrificed for terminal collection. From these animals, 200 μ L of systemic blood was collected via cardiac puncture, the spleen was isolated, as well as bone marrow extracted from 1 femur. The blood was processed as described above. Spleens were mechanically disrupted through a 70 μ m cell strainer, received ACK lysis for 3 minutes to lyse RBC, and were collected through a 70 μ m cell strainer again. Bone marrow (BM) was flushed through one femur and collected through a 70 μ m cell strainer. An aliquot of each processed tissue suspension was isolated for analysis of cell count (Celleca Cell Counter, Nexelom) and the remainder was used for phenotype assessment by flow cytometry (BD

Fortessa). For phenotypic assessment, samples were stained with antibodies specific for CD3 (BD), mouse CD45, CD25 (BD), FoxP3, CD4, CD8, IL15 (Lake Pharma), KLRG1, CD127, CD45RA, CD45RO, CD95, CD69, CCR7, CD56, and biotinylated IL15RaFc (ACROBiosystems). Antibodies were conjugated to FITC, PE, PE-Cy5, PE-Cy7, PerCP-Cy5.5, DyL650, APC-Cy7, BUV395, BUV737, BV421, BV510, BV605, BV711, or BV786 (Anti-human antibodies, all Biolegend, unless otherwise identified). In addition, a viability dye (e780 fixable viability dye, Invitrogen) was included for all samples. Samples were run on the BD Fortessa flow cytometer and analysis conducted using Flow Jo V10.7.1. To enumerate TILs throughout the study, TILs were gated as live cells, followed by lymphocytes, followed by human CD3+ and mouse CD45- cells. As demonstrated in FIG. 8B and FIG. 8C, transduced TILs were identified at high levels in periphery lymphoid organs on day 14 as well as day 53 post-infusion, and ACZ-treated regulated mbIL15 TILs demonstrated significantly higher persistence than their vehicle-treated counterparts ($p < 0.005$).

[00148] Table 5 shows viral vector sequences for the various constructs described herein.

Table 5: Viral vector sequences

Viral Vector	NA Sequence
OT-EGFP-001	SEQ ID NO:52 g c g c g c t c a c t g g c g t c g t t t t a c a c g t g t g a c t g g g a a a c c c t g g c g t t a c c c a a c t t a a t c g c c t t g c a g c a c a t e c c c c t t t c g c c a g c t g g c g t a a t a g c g a a g a g g c c c g c a c c g a t c g c c t t c c c a a c a g t t g c g c a g c c t g a a t g g c g a a t g g g a c g c g c c c t g t a g c g g c g c a t t a a g c g c g g g t g t g t g t t a c g c g c a g c g t g a c c g t a c a c t t g c c a g c g c c c t a g c g c c g t c c t t t c g t t t c c c t t c c t t c t t c t c g c c a c g t t c g c g g c t t t c c c g t c a a g c t c t a a t e g g g g c t c c c t t a g g g t t c c g a t t a g t g t t a c g g c a c c t c g a c c c c a a a a a a c t g a t t a g g t g a t g g t c a c g t a g t g g c c a t c g c c c t g a t a g a c g g t t t t c g c c t t t g a c g t t g g a g t c c a c g t t c t t a a t a g t g g a c t c t t g t t c c a a a c t g g a a c a c a c t c a a c c c t a t c g g t c t a t t c t t t g a t t t a a g g g a t t t g c c g a t t t c g g c c t a t t g g t t a a a a a t g a g c t g a t t a a c a a a a t t a a c g c g a a t t t a c a a a a t a t t a a c g c t t a c a a t t a g t g g c a c t t t c g g g a a a t g t c g c g g a a c c c c t a t t t g t t a t t t t c t a a t a c a t t c a a a t a t g t a t c c g c t c a t g a g a c a a a c c c t g a t a a t g c t t c a a t a a t t g a a a a a g g a a g a g t a t g a g t a t t c a a c a t t t c c g t g t c g c c c t a t t c c c t t t t t g c g g c a t t t g c c t c c t g t t t t g t c t a c c c a g a a a c g t g t g t g a a a g t a a a g a t g c t g a a g a t c a g t t g g g t g c a c g a g t g g g t a c a t c g a a c t g g a t c a a c a g c g g t a a g a t c c t t g a g a g t t t c g c c c c g a a g a c g t t t c c a a t g a t g a g c a c t t t a a g t t c t g c t a t g t g g c g g t a t t a t c c c g t a t t g a c g c c g g g c a a g a g c a a c t c g g t c g c c g c a t a c a c t a t t c t c a g a a t g a c t t g g t g a g t a c t c a c a g a a a g c a t c t t a c g g a t g g c a t g a c a g t a a g a a t t a t g c a g t g c t g c c a t a a c c a t g a g t g a t a a c a c t g c g g c c a a c t a c t t c t g a c a a c g a t c g g a g g a c c g a a g g a g c t a a c c g c t t t t t g c a c a a c a t g g g g a t c a t g t a a c t g c c t t g a t c g t t g g g a a c c g g a g c t g a a t g a a g c c a t a c c a a a c g a c g a g c g t g a c a c c a c a g a t g c c t g t a g c a a t g g c a a c a c g t t g c g a a a c t a t t a a c t g g c g a a c t a c t a c t a g c t t c c c g g c a a c a a t t a a t a g a c t g g a t g g a g g c g g a t a a a g t t g c a g g a c c a t t c t g c g t c g g c c c t c c g g t g g t g t t a t t g t g a t a a a t c t g g a g c c g t g a g c g t g g g t c t c g g g t a t c a t t g c a g c a c t g g g c c a g a t g g t a a g c c c c c g t a t c g t a t c a c a c g a c g g g a g t c a g g c a a c t a t g g a t g a a c g a a a c t t a t t a a a a g g a t c a g t g a a g a t c c t t t t g a t a a t c a t g a c c a a a a t c c c t a a c g t a g t t t c g t c c a c t g a g c g t c a g a c c c c g t a g a a a g a t c a a a g a t c t t c t t g a t c c t t t t t c t g c g c g t a a t c g t c t t g c a a c a a a a a a c c a c c

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Example 9. Rapid Expansion Protocol With Retroviral Transfection of TILs

[00149] Pre-REP TILs were prepared similarly to that of Example 1. Briefly, Melanoma and head and neck tumor samples were obtained from Cooperative Human Tissue Network. Tumor samples were cut into 1-3 mm fragments in Hanks' Balanced Salt Solution (HBSS) buffer and fragments were placed in Grex vessels at 1-10 fragments/flask in TIL culture media (RPMI-1640 supplemented with GlutaMAX (Thermo Fisher), 1% Penicillin/Streptomycin, 1 mM Sodium Pyruvate, 1% HEPES, 50 μ M 2-Mercaptoethanol (Invitrogen) and 10% heat-inactivated human AB serum (Valley Bio)) containing 6000 IU/mL IL2 (Peprotech), 10ug/mL 41BB antibody (Creative BioLabs), 30ng/mL of CD3 antibody (OKT3, Biolegend), and 0.1 mg/mL Normocin (InvivoGen). Vessels were routinely fed when nutrient depletion was identified, roughly every 3-4 days. This culture process is referred to as pre-rapid expansion protocol (pre-REP). After pre-REP, TILs were aliquoted, frozen in cell freezing media (BamBanker, Bulldog Bio or Cryostor-10, STEMCELL Technologies) and stored long-term in liquid nitrogen.

[00150] These pre-REP TILs were thawed and rested overnight in TIL media with 6000 IU/mL human IL2. TILs were then activated for 24 hr in 24-well plates coated with OKT3 at 3 μ g/mL (Ultra-LEAF purified anti-human CD3 antibody, Biolegend) and 6000 IU/mL human IL2. RetroNectin (30 μ g/mL) was used to coat 24-well non-tissue culture cell culture plates overnight at 4°C. The following day, RetroNectin was removed, the plates were blocked with 2% bovine serum albumin (BSA) in PBS, and the plates were then washed with PBS. Gibbon Ape Leukemia Virus (GALV) pseudotyped gamma retroviral vector (where mbIL15-CA2 DRD expression is under control of a promoter derived from murine leukemia virus LTR) supernatants were prepared from a stable producer cell line. Retroviral vector supernatant was diluted in TIL media and added in a total volume of 500 μ L per well resulting in an approximate MOI of 16-80. The plates containing viral vector were centrifuged at 1400 $\times$ g for 2 hr at 32°C, and the supernatant was then removed. After supernatant removal, 1.0×10^6 activated TILs were transferred per well with 100 IU/mL IL2 and incubated at 37°C overnight. Cells were processed similarly without virus addition and used as negative control (“unengineered”). 24 hours after transduction, 5×10^5 TILs were transferred into each well of a 6M GREX well plate (Wilson Wolf) in a total of 60 mL TIL media per well (RPMI-1640 supplemented with GlutaMAX (Thermo Fisher), 1% Penicillin/Streptomycin, 1 mM Sodium Pyruvate, 1% HEPES, 50 μ M 2-Mercaptoethanol (Invitrogen) and 10% heat-inactivated human AB serum (Valley Biomed)). Irradiated K562 feeder cells (transduced with 4-1BBL and mbIL21 and irradiated at 100Gy) or irradiated PBMC feeder cells (irradiated at 25Gy) were thawed and added to the culture at a ratio of 50:1 K562:TILs or 200:1 PBMC:TILs, respectively. TILs transduced with the regulated mbIL15 construct received 25 μ M Acetazolamide (SelleckChem) and untransduced TILs received 6000 IU/mL IL2. The cells were grown for 14 days in the GREX plates for the “rapid expansion protocol” or REP, and media was added or replaced as necessary.

Example 10. Regulated mbIL15 modified TILs: signaling and polyfunctionality

ACZ regulates IL15 expression and signaling in regulated mbIL15 TILs in a dose-dependent fashion.

[00151] Pre-REP TILs were prepared similarly to the methods of Example 1-3 and 9, and unengineered and mbIL15 TIL generated accordingly as described in Examples 1-3 and 9. Engagement of the IL15 signaling pathway results in phosphorylation of signal transducers downstream, including the transcription factor protein STAT5 and ribosomal protein S6. To

demonstrate that ACZ-regulated mbIL15 expression results in IL15 signaling in regulated mbIL15 TILs, a phospho-flow cytometry-based assay was employed as follows:

Cryopreserved regulated mbIL15 TILs obtained from four human donors (Patients 1-4), were thawed and then rest in ACZ-free media for 24 hours. Next, the regulated mbIL15 TILs were regulated for 18 hours in the presence of a range of concentrations of ACZ including 0.1, 1, 2.5, 5, 10, 25, 100 μ M, as well as vehicle control. The regulated mbIL15 TILs were then collected for staining and FACS analysis.

[00152] Briefly, cells were stained using antibodies for CD3, CD4, CD8, IL15 and a Live/Dead marker. Then cells were fixed in 2% formaldehyde (BD Cytofix) and permeabilized using a methanol-based buffer (BD Phospho Perm III Buffer) before staining with antibodies specific for phosphorylated STAT5 (Biolegend) and S6 (Cell Signaling Technology). Cells were acquired on the BD Symphony and analyzed using FlowJo software.

[00153] With increasing concentrations of ACZ expression of mbIL15 also increases, plateauing at around 10-25 μ M of ACZ. FIG. 9A. Similarly, the staining intensity of pSTAT5 and pS6 increased with higher concentrations of ACZ in regulated mbIL15 TILs, indicative of a greater degree of IL15 signaling. These results show a dose-dependent relationship between ACZ and IL15 expression and signaling. FIGs. 9B-E and FIG. 10.

Constitutive mbIL15 expression and ACZ regulation of regulated mbIL15 TILs engage the IL15 signaling pathway

[00154] In order to compare different strategies for IL15 expression, TILs were utilized that constitutively express mbIL15 and regulated mbIL15 TILs. Cryopreserved unengineered TILs, constitutive mbIL15 TILs, and regulated mbIL15 TILs, from three human donors were thawed and then rested in ACZ-free media for 24 hours. Next, the foregoing TILs were regulated in culture media for 18 hours, as follows: (1) 200 IU/mL of IL2 (Peprotech) was added to unengineered TILs; and (2) 25 μ M ACZ was added to regulated mbIL15 TIL cultures. Vehicle was added to control conditions. After the 18-hour treatment, the cells were stained using antibodies for CD3, CD4, CD8, IL15 and a Live/Dead marker. Then, cells were fixed in 2% formaldehyde (BD Cytofix) and permeabilized using a methanol-based buffer (BD Phospho Perm III Buffer) before staining with antibodies specific for phosphorylated STAT5 (Biolegend) and S6 (Cell Signaling Technology). Cells were acquired on the BD Fortessa and analyzed using FlowJo software.

[00155] As shown in FIG. 11, IL2 shares an overlapping signaling pathway with IL15, including signaling through STAT5 and S6. Unengineered TILs cultured with IL2 showed increased engagement of the signaling pathway compared to the corresponding vehicle condition. FIG. 11. Similarly, both constitutive mbIL15 expression and regulated mbIL15 TILs +ACZ displayed increased phosphorylation of the STAT5 and S6 compared to the regulated mbIL15 TILs +vehicle control. FIG. 11.

Regulated mbIL15 TILs demonstrates greater polyfunctionality than unengineered TILs + IL2

[00156] Polyfunctional T cells have the capacity to produce multiple effector molecules simultaneously in response to a stimulus. Additionally, polyfunctionality is correlated with T cell efficacy. To compare polyfunctionality of unengineered TILs to regulated mbIL15 TILs, cryopreserved cells were thawed and allowed to rest in IL2- and ACZ-free media for 24 hours. Next, regulation of the cells occurred as follows: unengineered TILs were regulated for 18 hours in the presence of a range of concentrations of IL2 (20, 200, 1000 and 6000 IU/mL, or vehicle); regulated mbIL15 TILs were regulated in the presence of ACZ (0.1, 1, 5, 10, 25, 100 μ M ACZ, or vehicle) for 18 hours. Then, cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Biolegend) in the presence of brefeldin A (Biolegend) and monensin (Life Technologies Corporation). Unstimulated unengineered TILs and unstimulated regulated mbIL15 TILs were used as a control. After stimulation, cells were then collected for staining and FACS analysis.

[00157] Briefly, cells were stained using antibodies for CD3, CD4, CD8, IL15 and a viability dye. Then, cells were formaldehyde-fixed and permeabilized (BD Cytofix/Cytoperm kit), then stained using antibodies for TNF α and IFN γ (Biolegend). Cells were acquired on the BD Fortessa and analyzed using FlowJo software. Cells that are double-positive for expression of TNF α and IFN γ are considered polyfunctional.

[00158] As shown in FIG. 12, while all culture conditions contained some polyfunctional populations, polyfunctionality in regulated mbIL15 TILs increased with higher concentrations of ACZ. FIG. 12A, 12B. Additionally, regulated mbIL15 TILs were more polyfunctional than unengineered TILs + IL2 from the same donor. FIGs. 12A, 12C. The percent of regulated mbIL15 TILs expressing mbIL15 also displayed a dose-response relationship with ACZ dose.

Example 11. *In vivo* efficacy of regulated IL15 TILs**PDX163A efficacy**

[00159] A patient-derived xenograft (PDX) model was created from a fresh primary melanoma sample (Patient tumor No. M1200163A) acquired from a tumor bank (Cooperative Human Tissue Network: CHTN). A mouse model was established using NSG female mice (Jackson Laboratory; Catalog No. 000557). Once the model was established, cryopreserved sections of tumor were aseptically implanted into isoflurane-anesthetized, immune-compromised mice (NSG female mice; Jackson Laboratory; Catalog No. 000557). Tumors were allowed grow to approximately 1000 mm³ – 2000 mm³ and the mice were then euthanized. The tumors were aseptically collected, sectioned into ~100 mg sections, and then implanted into a larger cohort of mice that were allowed to grow for 13 days. After 13 days, the tumors were measured and randomized (50 mm³ – 100 mm³) into respective treatment groups. On the next day, 10 million (10M) TILs were introduced intravenously. TILs were generated according to the rapid expansion protocol (REP) described above.

[00160] Treatment groups were as follows: (1) unengineered TILs dosed with IL2; and (2) regulated mbIL15 TILs dosed with acetazolamide (ACZ). Mice receiving unengineered TILs were dosed twice daily with 50,000 International Units (IUs) of IL2 for 5 days. Mice treated with regulated mbIL15 TILs received either vehicle or 200 mg/kg acetazolamide (ACZ) daily, for the entire study. Tumors and body weights were collected twice weekly.

[00161] FIG. 13 shows the results of a patient-derived xenograft (PDX) model. At the end of the end of the rapid expansion protocol (REP), unengineered TILs and regulated mbIL15 TILs (+/- acetazolamide (ACZ)) were adoptively transferred into mice bearing a human melanoma PDX. Mean tumor volumes were evaluated (+/- SEM). FIG. 13A shows mean tumor volume for a given treatment at days post adoptive cell transfer (ACT). FIG. 13B shows tumor volume at days post ACT for no TILs (top left); unengineered TILs + IL2 (top right); regulated mbIL15 TILs + vehicle (bottom left); and regulated mbIL15 TILs +ACZ (bottom right). As shown in FIG. 13, regulated mbIL15 TILs + ACZ significantly superior anti-tumor efficacy compared to unengineered TIL + IL2.

SK-MEL-1 efficacy

[00162] A SK-MEL-1 xenograft cancer model was created to evaluate regulated mbIL15 TILs of the present invention. Cells obtained from the thoracic duct of a patient with

widespread and rapidly progressing malignant melanoma (ATCC Catalog No. HTB-67) were used to create the model. NSG female mice (Jackson Laboratory; Catalog No. 000557) were the mice used to receive the cancer cells. Briefly, low passage cells were thawed and grown to scale maintaining viable, sub-confluent cultures. On the day of injection, cells were counted, washed, and resuspended in sterile PBS at a concentration of 30×10^6 cells/mL (3^6 cells per injection of 100 μ L). Each mouse received 100 μ L of cells injected subcutaneously on the shaved right flank using a BD tuberculin syringe, containing a 27 gauge, $\frac{1}{2}$ inch needle. Tumors were allowed to grow for 9 days, and were then measured and randomized ($50 \text{ mm}^3 - 100 \text{ mm}^3$) into their respective treatment groups. On the next day, 10 million (10M) TILs were introduced intravenously. TILs were generated according to the rapid expansion protocol (REP) described above.

[00163] Treatment groups were as follows: (1) unengineered TILs dosed with IL2; and (2) regulated mbIL15 TILs dosed with acetazolamide (ACZ). Mice receiving unengineered TILs were dosed twice daily with 50,000 International Units (IUs) of IL2 for 5 days. Mice treated with regulated mbIL15 TILs received either vehicle or 200 mg/kg acetazolamide (ACZ) daily for the entire study. Tumors and body weights were collected twice weekly.

[00164] FIG. 14 shows the results of a SK-MEL-1 xenograft cancer model. At the end of the end of the rapid expansion protocol (REP), unengineered TILs and regulated mbIL15 TILs (+/- acetazolamide (ACZ)) were adoptively transferred into mice bearing SK-MEL-1 tumors. Mean tumor volumes were evaluated (+/- SEM). FIG. 14A shows mean tumor volume for a given treatment at days post adoptive cell transfer (ACT). FIG. 14B shows tumor volume at days post ACT for no TILs (top left); unengineered TILs + IL2 (top right); regulated mbIL15 TILs + vehicle (bottom left); and regulated mbIL15 TILs + ACZ (bottom right). As shown in FIG. 14, the results demonstrate regulated mbIL15 TILs + ACZ show significantly superior anti-tumor efficacy compared to unengineered TIL + IL2.

Example 12. *In vitro* cytotoxicity with regulated mbIL15 TILs

[00165] Pre-REP TILs were prepared similarly to the methods of Example 1-3 and 9, and unengineered and mbIL15 TIL generated according to the methods of Examples 1-3 and 9. To evaluate the anti-tumor cytotoxic potential of regulated mbIL15 TILs, a tumor-TIL co-culture assay was performed, using the HLA-matched tumor cell line SK-MEL-1 (ATCC) and six different patient TIL samples. Identical experiments were also set up using PDX cells. The patient TIL samples evaluated were expanded unengineered TILs, or expanded regulated

mbIL15 TILs. The regulated mbIL15 TILs were created according to the REP protocol described above (Examples 1-9), and then cryopreserved. Unengineered TILs and regulated mbIL15 TILs from the six patients were then thawed, counted, and rested at a cell density of 7.5×10^5 cells/mL for 24 hours in culture media supplemented with either: +/- 6000 IU/mL IL2 for unengineered TIL, or vehicle (DMSO); or 25 μ M ACZ for regulated mbIL15 TILs. The following day, HLA-matched SK-MEL-1 cells were harvested from *in vitro* culture, and labeled with Cell Trace Far Red, according to the manufacturer's protocol. Additionally, PDX cells were obtained from fresh or cryopreserved chunks and digested with GentleMACs (Miltenyi) according to manufacturer's protocol.

[00166] The TILs were then co-cultured at 5:1, and 1:1 (TIL effector:tumor target) ratios with the labeled melanoma cells in the same supplemented IL2 or ACZ conditions listed above, with or without MHC Class I blocking reagent (tumor cells alone cultured with 80 μ g/mL of anti-human HLA ABC for 2 hours prior to co-culture with TILs). Additional controls of unlabeled and labeled melanoma cells alone were included to assess background caspase-3 activity in the co-culture system. This TIL-tumor cell co-culture was incubated for 3 hours, after which the cells were fixed, permeabilized, and stained for intracellular cleaved caspase-3 (a marker for irreversible commitment to cell death within tumor cells).

[00167] Samples were acquired on the BD Fortessa flow cytometer with analyses conducted using Flow Jo V10.7.1, where cytotoxicity was determined by the percentages of cells staining positive for cleaved caspase-3 within the population of live, Cell Trace Far Red positive cells (subtracting the background caspase-3 positivity).

[00168] As shown in FIG. 15, in this assessment of anti-tumor cytotoxicity of TIL-tumor pairs, regulated mbIL15 TILs exhibited superior anti-tumor cytotoxic activity across all 6 donors, compared to unengineered TILs + IL2. FIG. 15.

Example 13: Generation of unengineered and mbIL15 TIL with distinct feeder cells

[00169] Pre-REP TILs generated from tumor samples were prepared as described in Example 1 and 9. Pre-REP TILs were thawed and rested for 48-hours in TIL media (RPMI-1640 supplemented with GlutaMAX (Thermo Fisher), 1% HEPES, 50 μ M 2-Mercaptoethanol (Invitrogen) and 10% heat-inactivated human AB serum (Valley Bio) with 6000 IU/mL human IL2 (PeproTech). TILs were then activated for 24 hr in 24-well NUNC plates coated with anti-CD3 (OKT3, Miltenyi Biotec) at 3 μ g/ and 6000 IU/mL soluble human IL2. RetroNectin (30 μ g/mL) was used to coat 24-well non-coated cell culture plates

overnight at 4°C. The following day, RetroNectin was removed, the plates were blocked with 2.5% human serum albumin (HSA) in PBS, and the plates were then washed with PBS. BaEV-pseudotyped lentiviral supernatants, prepared as described in Example 9, were diluted in TIL media and added to each well to achieve an MOI of 0.01 – 0.6. The plates containing viral vector were centrifuged at 1400g for 2 hr at 32°C, and the supernatant was then removed. After supernatant removal, 1×10^6 activated TILs were transferred per well with 0 - 100 IU/mL IL2 and incubated at 37°C overnight. Cells were processed similarly without virus addition into TIL media and used as a negative control (“unengineered”). Twenty-four hours after transduction, TILs were transferred into 6M GREX flasks (Wilson Wolf) into a total of 40 mL TIL REP media (50% TIL media as described above, 50% AIM-V media (Gibco). Proliferation-impaired (irradiated or mitomycin-C treated) feeder cells (pooled PBMCs, unmodified K562 feeders, K562 modified to express membrane-bound IL21, K562 modified to express 41BBL, K562 modified to express 41BBL and membrane-bound IL21) were added to the culture at a ratio of 50:1 K562 to TIL. Groups designated to receive exogenous IL21 were dosed with 50ng/mL recombinant human IL21. TILs transduced with the regulated mbIL15 construct received 25 μ M Acetazolamide (Hikma) and unengineered TILs received 3000 IU/mL IL2. The cells were grown for 14 days in the GREX plates for the “rapid expansion protocol” or REP, and media was added as necessary.

Evaluation of TIL expansion in REP

[00170] Periodically during the expansion, each GREX well was resuspended and mixed thoroughly, and an aliquot was taken for cell counting using Acridine Orange/Propidium Iodide viability dye (Cellaca Cell Counter, Nexcelom) and flow cytometry staining. Samples were stained using antibodies CD3-BUV395 (BD), CD56-BV711 (Biolegend), CD4-BV605 (Biolegend), CD8-Alexa Fluor 700 (Biolegend), IL15RaFc-Biotin (ACRO Biosystems) with secondary Streptavidin-BV421 (Biolegend), and fixable viability dye eFluor 780 (Thermo Fisher). Samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1.

[00171] Total TIL expansion was determined by obtaining the total viable cell counts at specific time points throughout REP. FIG. 16 shows that for mbIL15 TILs, use of K562 feeder cells and receiving both IL-21 and 41BBL-mediated co-stimulation resulted in the maximal cell expansion in REP and PBMC feeder cells as well as K562 feeder cells without 41BBL supported only sub-optimal levels of TIL expansion in REP. In contrast, although

unengineered TIL expanded in the presence of IL2 using any of the feeder cells, PBMC feeder cells promoted the maximal expansion of unengineered TIL in REP.

[00172] IL15 expression was determined by the percent of cells staining positive for BV421-streptavidin within the population of live, CD3 positive, CD56 negative cells. In mbIL15 TILs generated with K562 feeder cells and receiving both IL-21 and 41BBL-mediated co-stimulation, the frequency of mbIL15⁺ TILs increased through the REP process, suggesting enrichment of the mbIL15-transduced subset within the engineered TIL cell cultures (FIG. 18). Likewise, maximal expansion of mbIL15⁺ TILs in REP occurred when either constitutive or regulated mbIL15⁺ TILs are generated using K562 feeder cells with both IL-21 and 41BBL-mediated co-stimulation (FIG. 19).

[00173] CD4:CD8 ratios were determined by a ratio of the percent of cells staining positive for CD4 (of live, CD3 positive, CD56 negative cells) to the percent of cells staining positive for CD8 (of live, CD3 positive, CD56 negative cells). Expanded mbIL15 TILs generated with K562 feeder cells and receiving both IL-21 and 41BBL-mediated co-stimulation were enriched for CD8⁺ cytotoxic effector cells, as indicated by their decreased CD4:CD8 ratio throughout REP (FIG. 20). In contrast, the CD4:CD8 ratio of mbIL15 TILs generated with pooled PBMC feeders, unmodified K562 feeders, or K562 feeders expressing 41BBL alone did not decrease during REP.

[00174] For evaluation of polyfunctionality, unengineered and mbIL15 TILs at the end of REP were co-cultured in a 96-well tissue culture treated round bottom plate with Immunocult CD3/CD28 stimulation (Stem Cell Technologies) as per manufacturer's protocol. After 1 hour of incubation, 1000x transport inhibitors were added (Monensin from eBiosciences, Brefeldin A from Biolegend), and the co-cultured was incubated at 37°C for 5 additional hours. After the incubation, samples were stained using the antibodies described above, then fixed and permeabilized using Cytfix/Cytoperm reagents (BD Biosciences). Intracellular staining was performed with antibodies against IL2-BV737 (BD), IFN γ -FITC (Biolegend), Perforin-PerCPCy5.5 (Biolegend), TNF α -PECF594 (Biolegend), granzymeB-Alexa Fluor 700 (Biolegend). Samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1. Polyfunctionality was determined as the percent of TNF α and IFN γ double positive cells, of live lymphocytes. mbIL15 TIL generated with K562 feeder cells expressing both membrane-bound IL-21 and 41BBL demonstrated enhanced polyfunctionality at the end of REP as compared to mbIL15 TILs generated with PBMC feeder cells or unmodified K562 feeder cells (FIG. 21).

Evaluation of *in vitro* TIL persistence in an antigen-independent survival assay

[00175] Post-REP TILs were assessed for *in vitro* persistence in an antigen-independent survival assay. At the end of REP, unengineered and mbIL15 TILs were rested in supplement-free conditions for 24 hours. The following day, unengineered cells were cultured in duplicate at 1×10^6 cells/well in a 24-well GREX plate either without cytokine support or with 6000IU/mL IL2, and mbIL15 TILs were cultured at the same density either with 25 μ M ACZ or with the identical volume of vehicle (DMSO). On day 0, 100 μ L of each well was sampled for TIL enumeration and phenotypic characterization, which was performed by cell count and staining with antibodies as described above. On day 4, cells were resuspended, 500 μ L of cells were removed and 500 μ L of media + treatment were added to each well to bring the culture volume up to 1000 μ L. On day 6, cells were resuspended, a 100 μ L aliquot was sampled and phenotyped, 400 μ L of cells were removed, and 500 μ L of media + treatment were added to each well to bring the culture volume up to 1000 μ L. On day 8, cells were resuspended, 500 μ L of cells were removed and 500 μ L of media + treatment were added to each well to bring the culture volume up to 1000 μ L. On day 10, cells were resuspended, a 100 μ L aliquot was sampled and phenotyped, and then cultures were terminated. Samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1. Expanded mbIL15 TILs generated with K562 feeder cells and receiving both IL-21 and 41BBL-mediated co-stimulation demonstrate improved persistence in a 10-day survival assay compared to mbIL15 TILs generated with PBMC feeder cells or K562 feeder cells that are unmodified or express mbIL-21 and 41BBL independently (FIG. 22).

Assessment of TCR diversity

[00176] To measure TCRV β sub-family diversity, unengineered and mbIL15 TILs at the end of REP were stained for flow cytometry using the Beta Mark TCR Vbeta Repertoire Kit (Beckman Coulter) following manufacturer's protocol. Samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1, and TCRV β subfamily distribution was assessed by evaluating the percent positive for each subfamily and displaying the data as an aggregate of all covered subfamilies. Both unengineered and mbIL15 TILs maintained diverse TCRV β subfamily distribution regardless of the feeder cells for expansion in REP (FIG. 23).

PD1 expression in mbIL15 TILs with both 41BBL and IL21-mediated signaling

[00177] To evaluate the level of TIL exhaustion, PD1 expression was determined. Samples were stained using antibodies CD3-BUV395 (BD), CD56-BV711 (Biolegend), CD4-BV605 (Biolegend), CD8-Alexa Fluor 700 (Biolegend), PD1-PECy7 (Biolegend), CD25-BUV737 (Biolegend), IL15RaFc-Biotin (ACRO Biosystems) with secondary Streptavidin-BV421 (Biolegend), and fixable viability dye eFluor 780 (Thermo Fisher). For intracellular staining, cells were first stained with surface antibodies listed above, and then cells were fixed and permeabilized using BD Cytofix/Cytoperm manufacturer's protocol. Permeabilized cells were then stained using the antibody FoxP3-FITC (Biolegend), and samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1. PD1 expression was determined by the percent of cells staining positive for PD1 within the population of live, CD3 positive, CD56 negative cells. As shown in FIG. 25, PD1 expression is highest in unexpanded mbIL15 TIL, and expansion of mbIL15 TILs with both 41BBL and IL21-mediated signaling produces TILs with near baseline expression of PD1.

Example 14: Phenotype changes in mbIL15 TILs during engineering and expansion as compared to pre-REP TILs (Frequencies of CD8+, CD4+, PD1+ and regulatory T cells)

[00178] Phenotyping was performed to compare pre-REP TILs (as described in Example 1) to engineered mbIL15 TILs (as described in Example 3). Pre-REP and post-REP TILs were phenotyped by flow cytometry using antibodies for CD3, CD4, CD8, and PD1 as described in Example 13. As shown in FIG. 25A, the frequency of CD8+ T cells is higher and the frequency of CD4+ T cells is lower for post-REP mbIL15 TILs as compared with corresponding pre-REP TILs from the same TIL donors, which is consistent with the results shown in FIG. 20 from Example 13. This increase in CD8+ T cells reflects an increase in cytotoxic effector cells as discussed and evaluated in Example 13. Likewise, as shown in FIG. 25B, the post-REP mbIL15 TILs express lower levels of PD1 than corresponding pre-REP TILs from the same TIL donors, which is consistent with the results shown in FIG. 24 from Example 13.

[00179] To detect the regulatory T cells (T_{reg} cells) in the expanded population of TILs, samples were stained using antibodies CD3-BUV395 (BD), CD56-BV711 (Biolegend),

CD4-BV605 (Biolegend), CD8-Alexa Fluor 700 (Biolegend), PD1-PECy7 (Biolegend), CD25-BUV737 (Biolegend), IL15RaFc-Biotin (ACRO Biosystems) with secondary Streptavidin-BV421 (Biolegend), and fixable viability dye eFluor 780 (Thermo Fisher). For intracellular staining, cells were first stained with surface antibodies listed above, and then cells were fixed and permeabilized using BD Cytofix/Cytoperm manufacturer's protocol. Permeabilized cells were then stained using the antibody FoxP3-FITC (Biolegend), and samples were run on the BD Fortessa flow cytometer and analysis conducted using Flow Jo V10.7.1. Regulatory T cells were identified as CD3⁺ T cells that are gated as CD4⁺ and further classified as CD25 and FoxP3 double positive cells. As shown in FIG. 25C, expanded mbIL15 TILs have a reduced proportion of regulatory T cells as compared to pre-REP TILs prior to the engineering step.

Example 15: Patient-derived xenograft (PDX) model and treatment with engineered TILs

Establishment of a patient-derived xenograft (PDX) model

[00180] A patient-derived xenograft (PDX) model (PDX 163A) was created from a fresh primary melanoma sample acquired from a tumor bank, as described in Example 11. Once the model was established, cryopreserved sections of tumor were aseptically implanted into isoflurane-anesthetized, immune-compromised mice. Tumors grew to approximately 1000 mm³ – 2000 mm³ upon when they were euthanized, and tumors were serially passaged into subsequent animals to maintain the PDX tumor growth and build cohorts of animals for efficacy studies (as described below).

[00181] The PDX163A tumors resected from the tumor-bearing animals were also assessed for their expression of shared melanoma tumor antigens using flow cytometry. To evaluate the level of conserved melanoma antigen on melanoma cells, the melanoma cell line A375 and melanoma PDX described herein were assayed by flow cytometry. Tumor chunk(s) from melanoma PDX as described in Example 11 were obtained fresh or from cryopreservation, and were digested with the GentleMACs (Miltenyi) according to manufacturer's protocol in order to obtain a viable single cell suspension. Samples were blocked with Fc blocking reagent and stained using antibodies against MART-1 (Biolegend), gp100 (Biolegend) and fixable viability dye eFluor 780 (Thermo Fisher). Samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1. The frequency of melanoma-associated antigen-expressing tumor cells was determined by the

percent of cells staining positive for either MART-1 or gp100, within the population of live cells. FIG. 26 shows that the conserved melanoma-associated antigens MART-1 and gp100 were both expressed on the PDX tumors selected for TIL efficacy modeling as described in this Example (below).

Selection of donors for allogeneic efficacy modeling

[00182] TILs from eight melanoma donors were generated as described in Examples 1-3 or 9. Briefly, after 3 weeks in the pre-REP culture, cryopreserved TILs were thawed and rested overnight with 6000 IU/mL human IL2. TILs were then activated with anti-CD3/CD28 Dynabeads or on OKT3-coated multi-well plates for 24 hours, after which point they were transduced with regulated mbIL15 vectors or unengineered. 24 hours after transduction, TILs were expanded with K562-IL21-41BBL feeder cells in GREX 6M well plates (Wilson Wolf) with 6000 IU/mL IL2 added to unengineered TILs, and 25 μ M acetazolamide (SelleckChem or Hikma) added to regulated mbIL15 TILs. After 14 days of expansion, TILs were harvested, de-beaded, and rested overnight with and without IL2 and acetazolamide.

[00183] Tetramer staining was used to determine which TIL donors were reactive to the shared melanoma antigens, MART-1 and gp100. To evaluate the level of antigen-reactive TILs, flow cytometry was performed to examine the frequency of tetramer-reactive cells. Samples were blocked with Fc blocking reagent and stained using antibodies CD3-BUV395 (BD), CD4-BV605 (Biolegend), CD8-Alexa Fluor 700 (Biolegend), HLA-A2:01-MART-1 tetramer (MBL International), HLA-A2:01-gp100 (MBL International), IL15RaFc-Biotin (ACRO Biosystems) with secondary Streptavidin-BV421 (Biolegend), and fixable viability dye eFluor 780 (Thermo Fisher). Samples were run on the BD Symphony flow cytometer and analysis conducted using Flow Jo V10.7.1. The frequency of antigen-reactive TILs was determined by the percent of cells staining positive for each of the two tetramers, independently, within the population of live, CD3 positive, CD8 positive cells. As shown in Figure X, all four of the donors tested demonstrated reactivity to MART-1 antigen, and three of four donors tested demonstrated reactivity to gp100 antigen. The tetramer positive populations indicate that the TILs contain a portion of cells that are reactive to the corresponding melanoma-associated antigens, through the HLA-A2:01 locus. In FIG. 27, donors indicated with a * were utilized in the PDX efficacy study as depicted in this Example (below).

[00184] Tumor chunk(s) from melanoma PDX as described in Example 11 were obtained fresh or from cryopreservation, and were digested with the GentleMACs (Miltenyi) according to manufacturer's protocol in order to obtain a viable single cell suspension. PDX cells were then resuspended in TIL media at 5×10^6 cells/mL. Ten $\mu\text{g/mL}$ mitomycin-C was added to the cells, which were then incubated for 30 minutes at 37°C . The cells were then washed three times with 50 mL TIL media. 1×10^5 PDX cells per well were added to a 96-well flat bottom tissue-culture treated plate. In some wells, 80 $\mu\text{g/mL}$ HLA-ABC (Biolegend) blocking antibody were added to block MHC class I on the target cells. TILs that were rested overnight were added at a 1:1 ratio of TIL:PDX for a total volume of 200 μL per well. As a positive control, TILs were co-cultured 1:1000 with PMA/ionomycin, which would elicit maximal $\text{IFN}\gamma$ secretion. As a negative control, TILs were co-cultured without any additional reagents or cells and identified as "Unstimulated" TIL. At a 24-hour time point, supernatant was saved from each well and the concentration of $\text{IFN}\gamma$ was assayed by MSD.

[00185] Figure 28 shows that interferon gamma ($\text{IFN}\gamma$) production after TIL:tumor cell co-culture can be used to predict TIL donors that are reactive to the PDX tumor. This *in vitro* assay demonstrates that TIL donors 006, 39A, and 41A are the donors with the highest amount of $\text{IFN}\gamma$ produced in response to the PDX, thus supporting their candidacy as donors to examine *in vivo* efficacy as described in this Example (below).

Use of a patient-derived xenograft (PDX) model for TIL efficacy studies

[00186] Tumors from PDX-tumor-bearing mice (passaged as described above) were aseptically collected, sectioned into ~ 100 mg sections, and then implanted into a larger cohort of mice that were allowed to grow for 13 days upon which being measured and randomized (50 mm^3 to 100 mm^3) into their respective treatment groups. On the next day, 10M TILs were introduced intravenously. Mice receiving unengineered TILs were dosed daily with 600,000 International units (IUs) IL2 for 4 days. Mice receiving the mbIL15 product in which mbIL15 was operably linked to CA2 received 200 mg/kg acetazolamide (ACZ) daily for the entire study. Tumors and body weights were collected twice weekly. The treatment paradigm is shown in FIG. 29. As shown in FIG. 30, the engineered TILs + ACZ showed superior anti-tumor effects as compared to unengineered TILs + IL2. Additionally, the engineered TILs, particularly in the presence of ACZ, showed better tumor infiltration as shown in FIG. 31A and greater numbers in both stroma and tumor compartments as shown in FIG. 31B.

WHAT IS CLAIMED IS:

1. A method of expanding tumor-infiltrating lymphocytes (TILs) engineered to express membrane-bound IL15, comprising culturing the TILs in the presence of modified K562 feeder cells.
2. The method of claim 1, wherein expansion occurs in the absence of exogenous IL2.
3. The method of claim 1 or 2, wherein the modified K562 feeder cells are replication incompetent.
4. The method of any one of claims 1-3, wherein the modified K562 feeder cells express a costimulatory molecule chosen from the tumor necrosis factor superfamily.
5. The method of claim 3, wherein the costimulatory molecule chosen from the tumor necrosis factor superfamily is 41BBL.
6. The method of any one of claims 1-5, wherein the K562 feeder cells express IL21 or IL7.
7. The method of claim 6, wherein the K562 feeder cells express membrane bound IL21.
8. A culture comprising
 - (a) T-cells or tumor infiltrating lymphocytes and
 - (b) modified K562 feeder cells, wherein the K562 feeder cells comprise a first exogenous nucleic acid sequence encoding a costimulatory molecule chosen from the tumor necrosis factor superfamily and a second exogenous nucleic acid sequence encoding IL21 or IL7.
9. The culture of claim 8, wherein the culture comprises modified tumor-infiltrating lymphocytes (TILs).

10. The culture of claim 9, wherein the TILs are modified to express membrane-bound IL15.
11. The culture of claim 10, wherein the expressed membrane-bound IL15 is operably linked to a drug responsive domain.
12. The culture of any one of claims 8-11, wherein the modified K562 feeder cells are replication incompetent.
13. The culture of any one of claims 8-12, wherein the modified K562 feeder cells express a membrane-bound IL21.
14. The culture of any one of claims 8-13, wherein the costimulatory molecule chosen from the tumor necrosis factor superfamily is 41BBL.
15. A method of expanding T cells or tumor infiltrating lymphocytes (TILs), comprising culturing the modified T cells or TILs in the presence of a population of modified K562 feeder cells, wherein the modified K562 feeder cells support expansion of T cells or TILs in the absence of IL2.
16. The method of claim 15, wherein the modified K562 feeder cells comprise a first exogenous nucleic acid sequence encoding a costimulatory molecule chosen from the tumor necrosis factor superfamily and a second exogenous nucleic acid sequence encoding IL21 or IL7.
17. The method of claim 15 or 16, wherein modified TILs are expanded.
18. The method of claim 17, wherein the TILs are modified to express membrane-bound IL15.
19. The method of claim 18, wherein the expressed membrane-bound IL15 is operably linked to a drug responsive domain.

20. The method of any one of claims 15-19, wherein the modified K562 feeder cells are replication incompetent.
21. The method of any one of claims 15-20, wherein the modified K562 feeder cells express a membrane-bound IL21.
22. The method of any one of claims 15-21, wherein the costimulatory molecule chosen from the tumor necrosis factor superfamily is 41BBL.
23. An expanded population of modified T cells made by the method of any one of claims 15-22.
24. An expanded population of TILs made by the method of any one of claims 1-7.
25. A method of treating cancer in a subject, comprising administering to the subject an expanded population of modified TILs, wherein the TILs are expanded according to the method of any one of claims 1-7.
26. The method of treating cancer of claim 25, wherein the subject is not administered exogenous IL2.

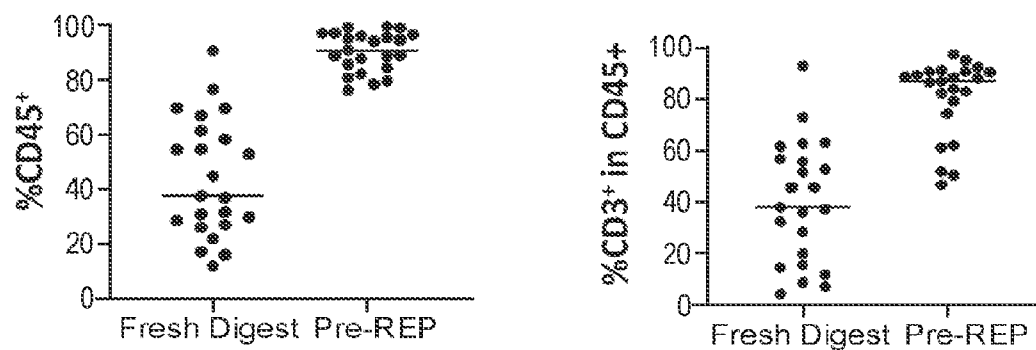


FIG. 1

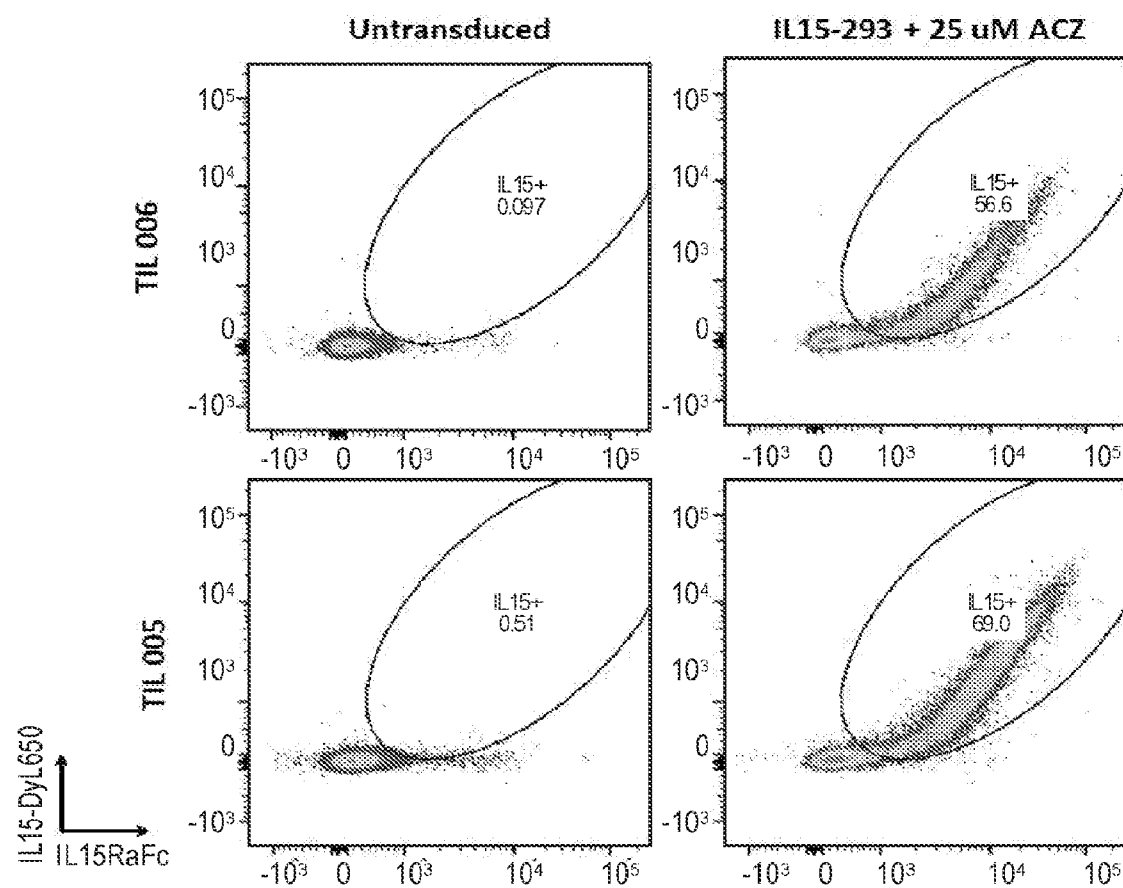


FIG. 2

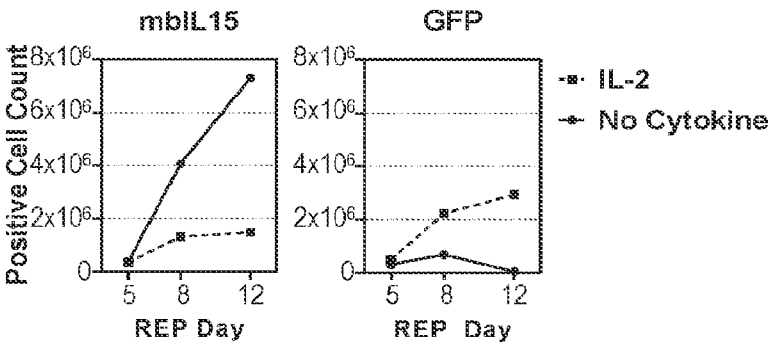


FIG. 3A

Post-REP Antigen-Independent Survival

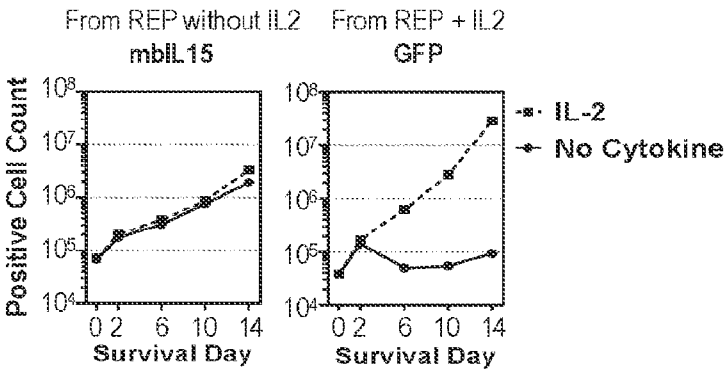


FIG. 3B

Post-REP Antigen-Independent Persistence

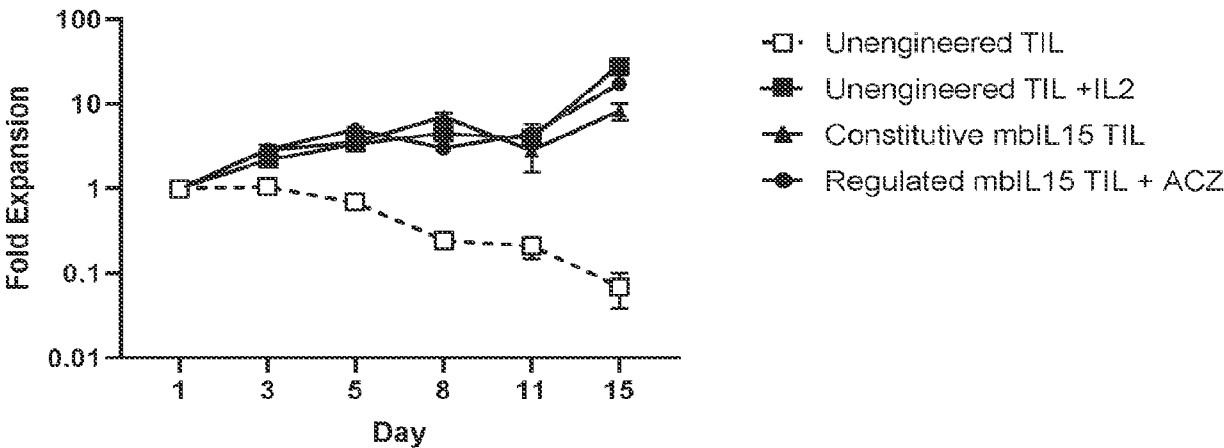


FIG. 4

TIL Persistence in Antigen(A375)-dependent survival assay

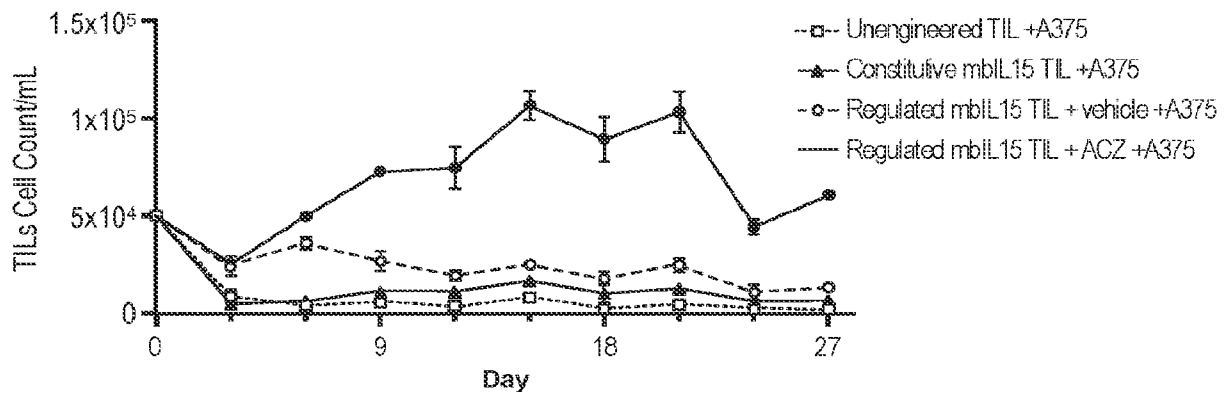


FIG. 5

Interferon gamma production

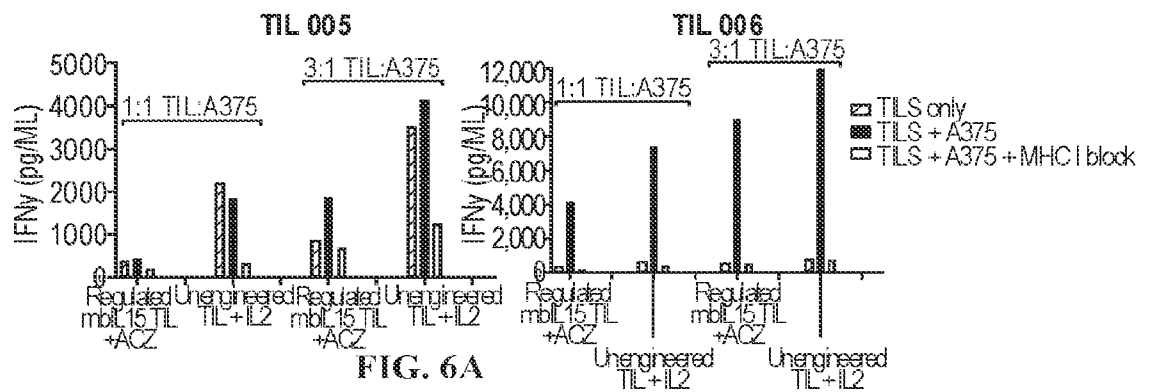


FIG. 6A

Cytotoxicity

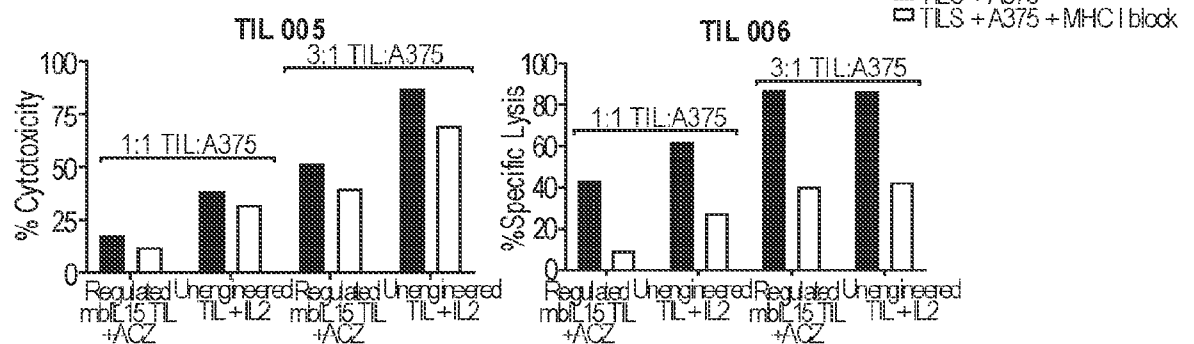


FIG. 6B

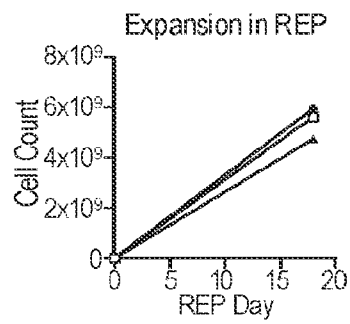


FIG. 7A

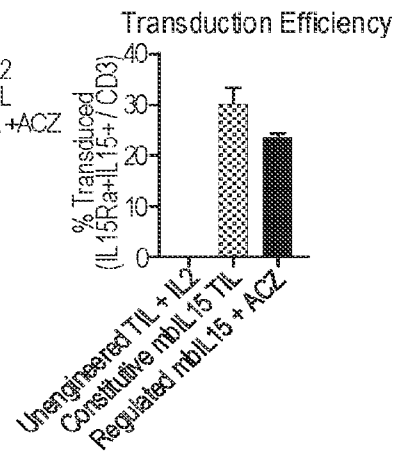


FIG. 7B

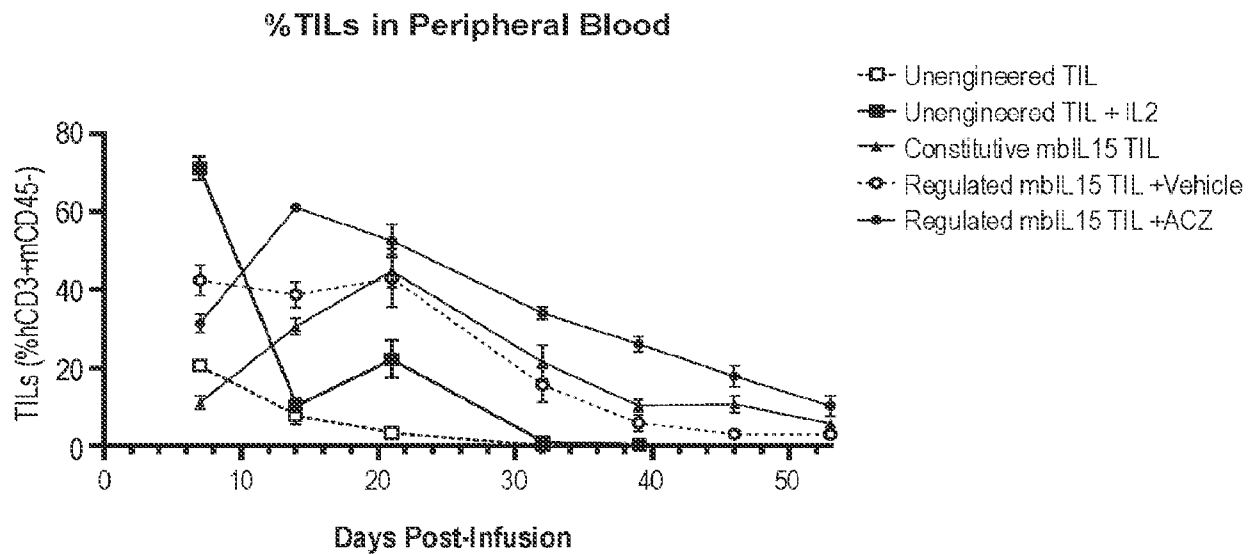


FIG. 8A

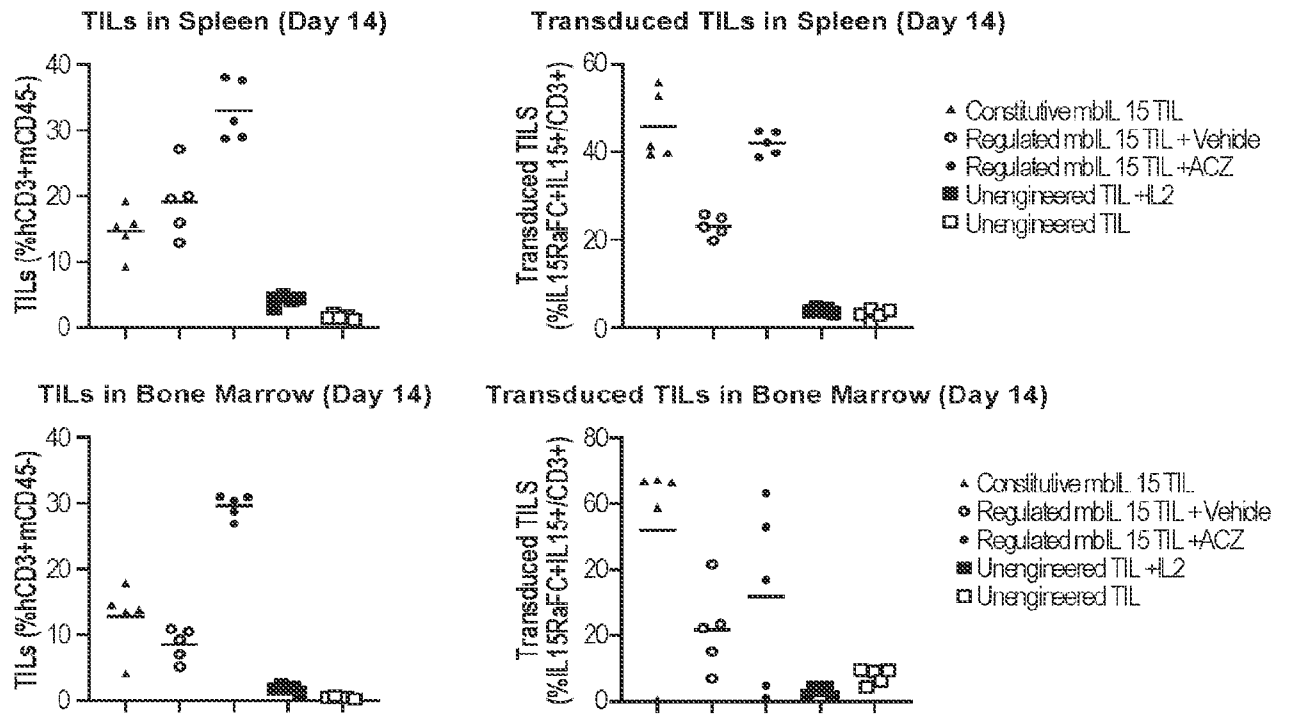
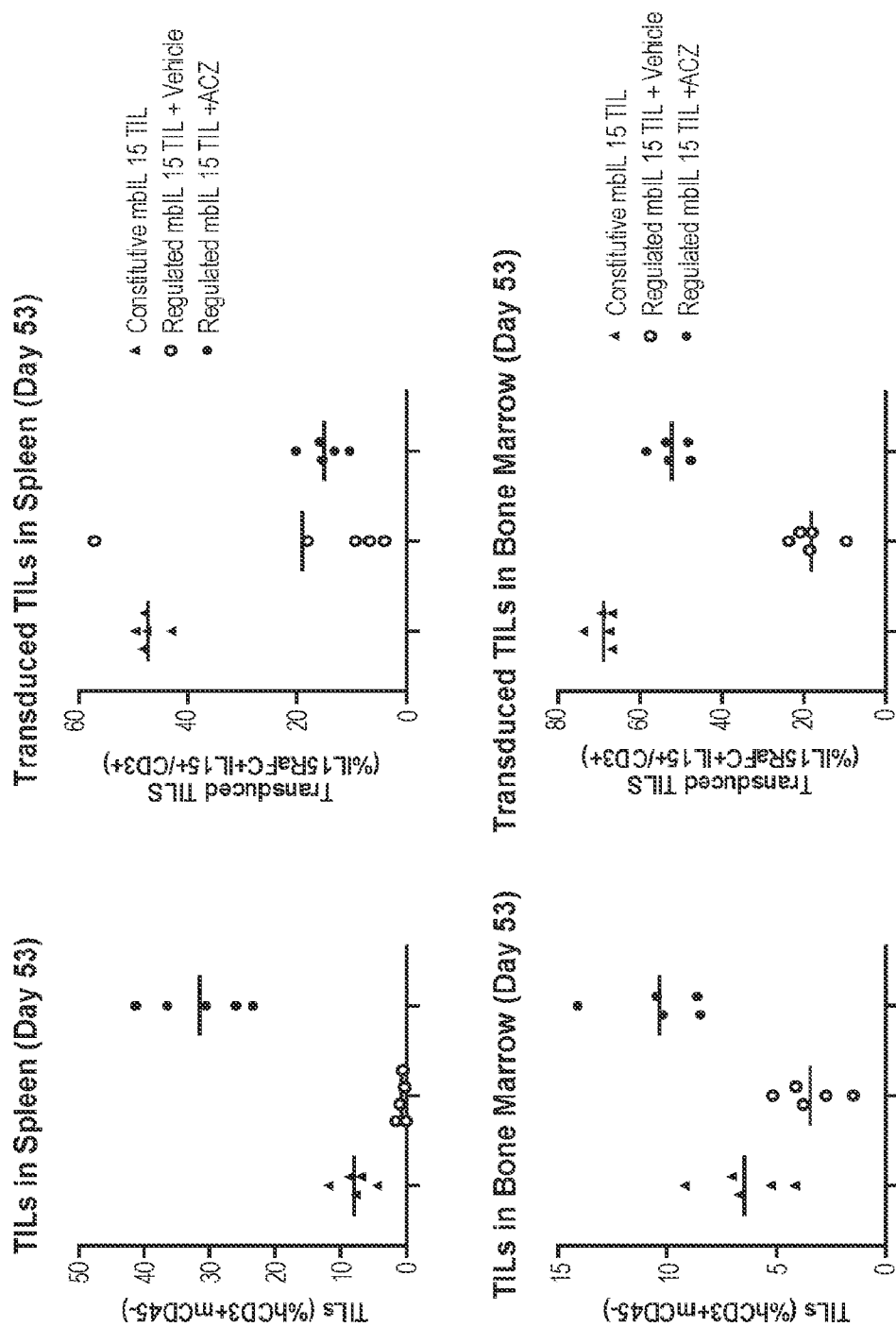


FIG. 8B



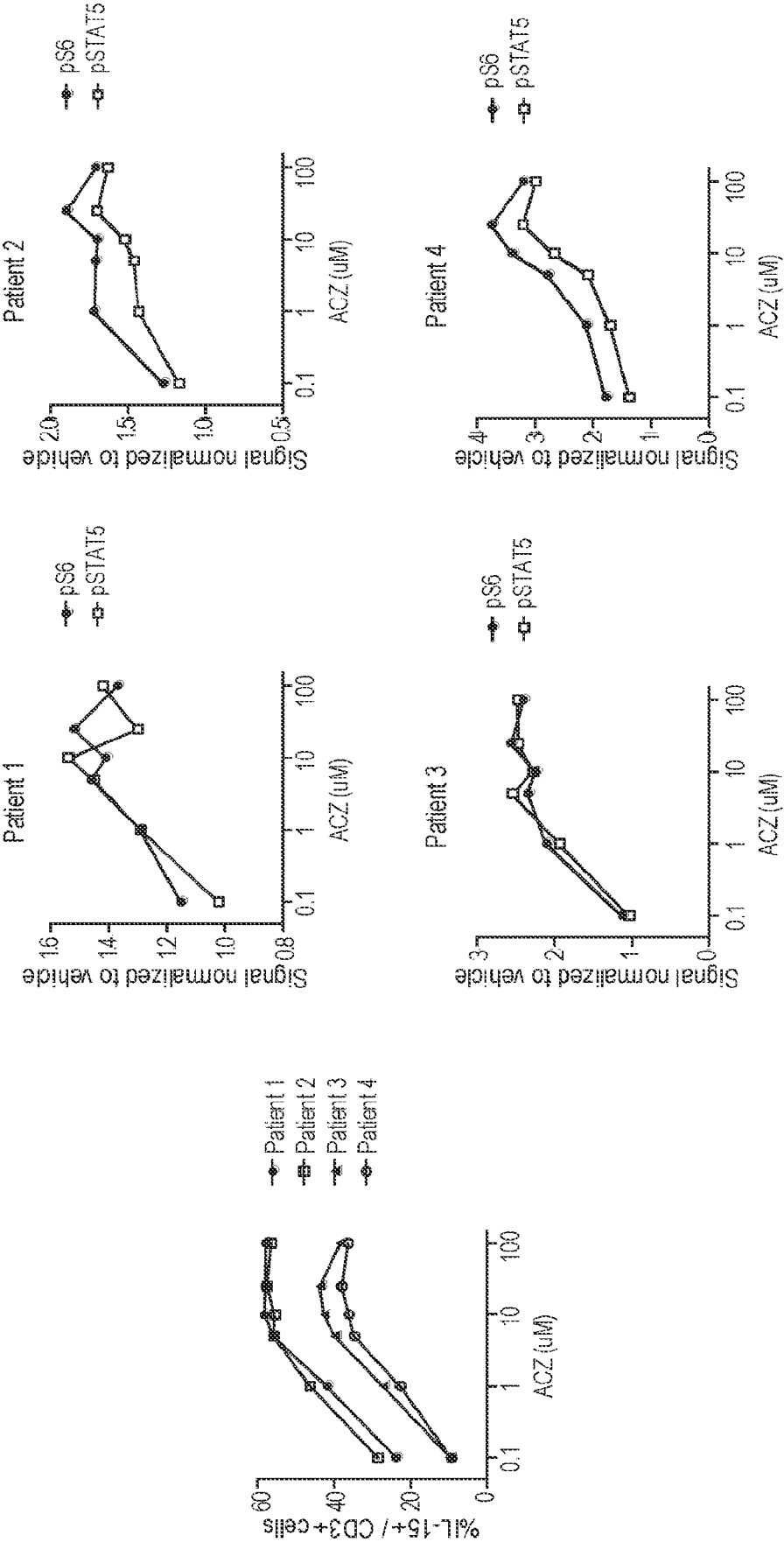


FIG. 9

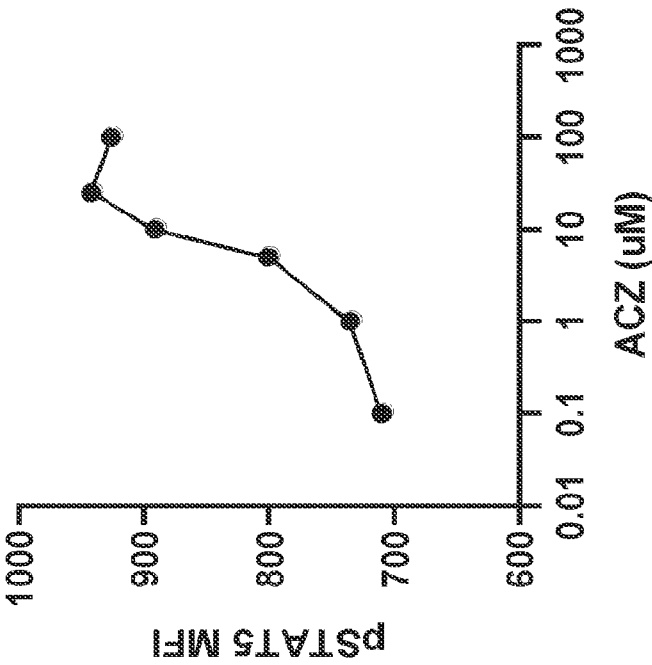
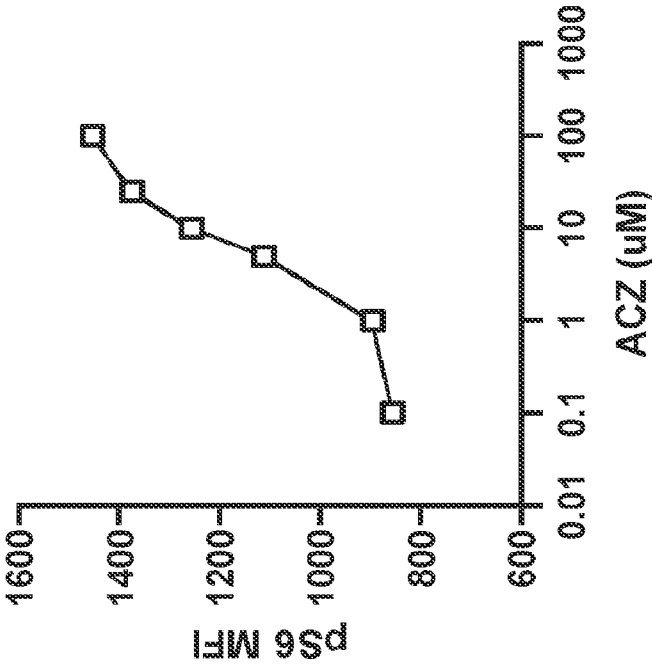


FIG. 10

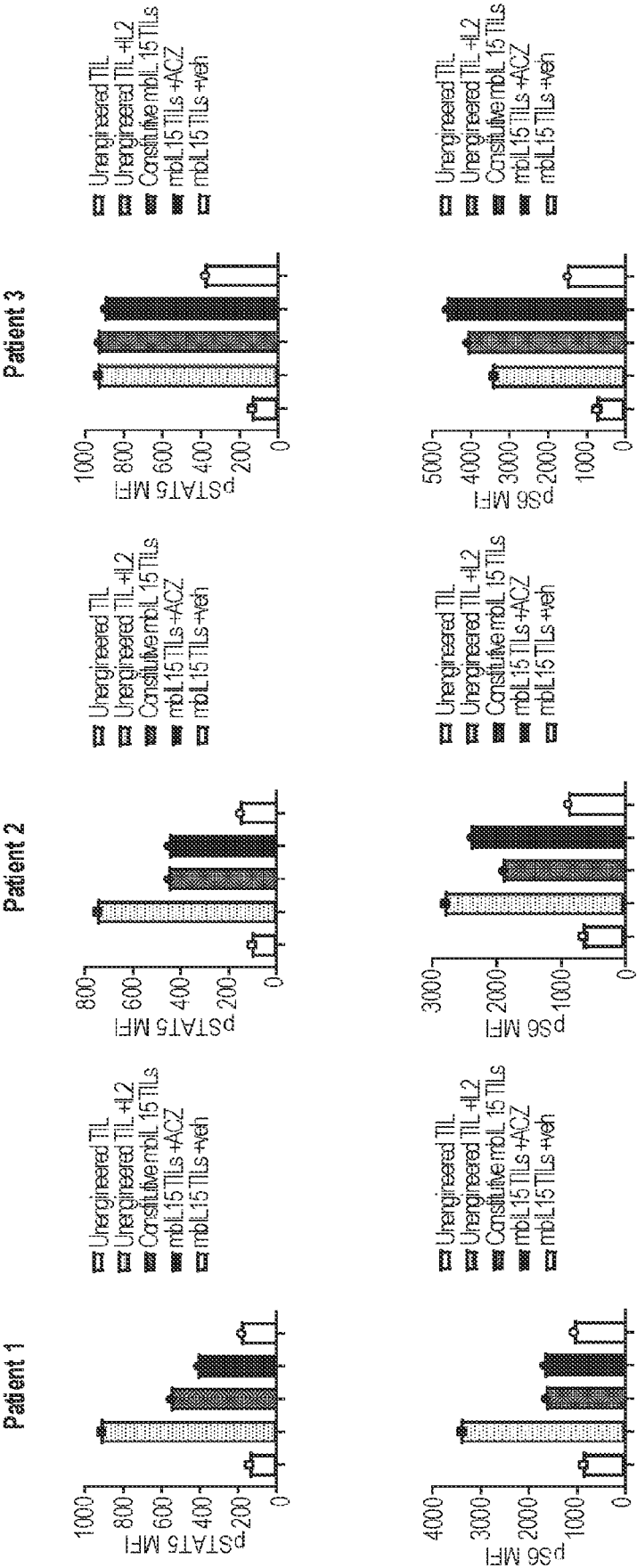


FIG. 11

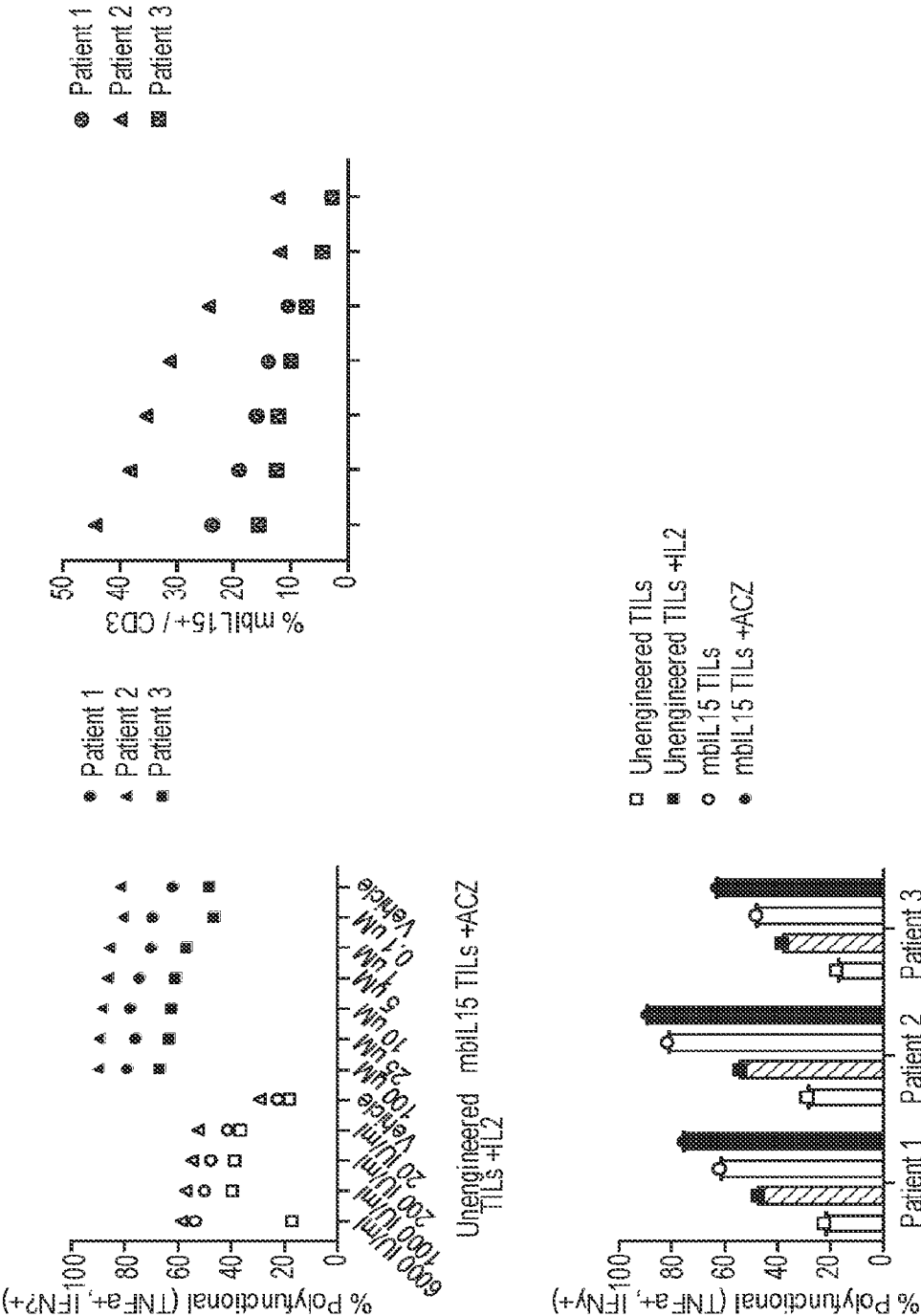


FIG. 12

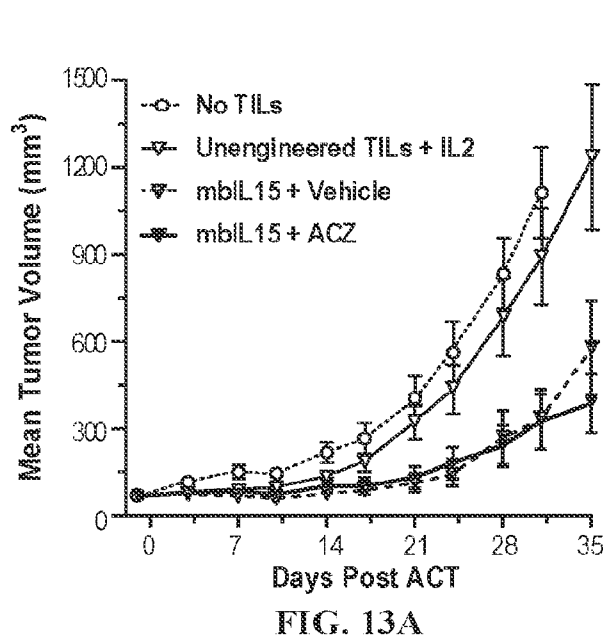


FIG. 13A

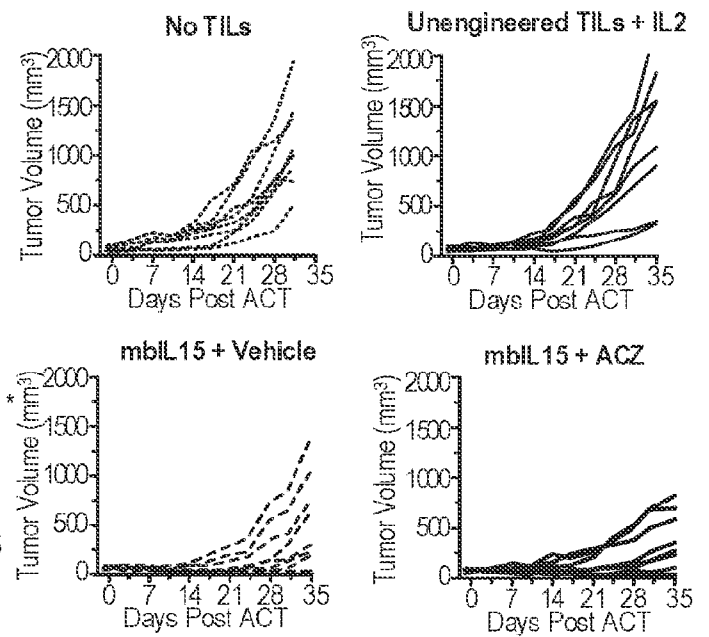


FIG. 13B

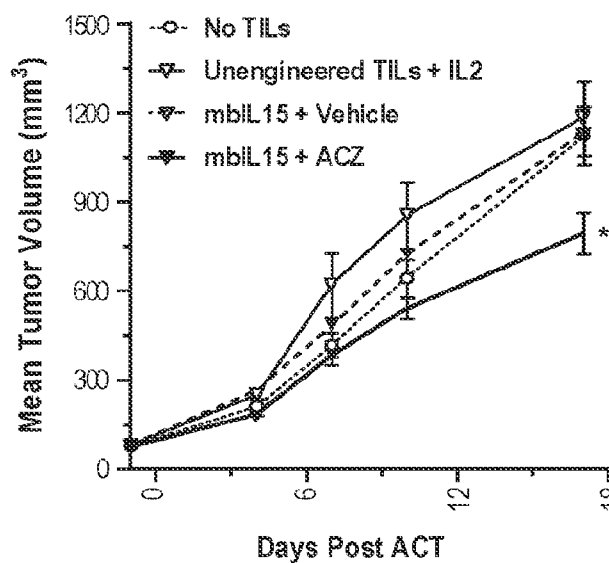


FIG. 14A

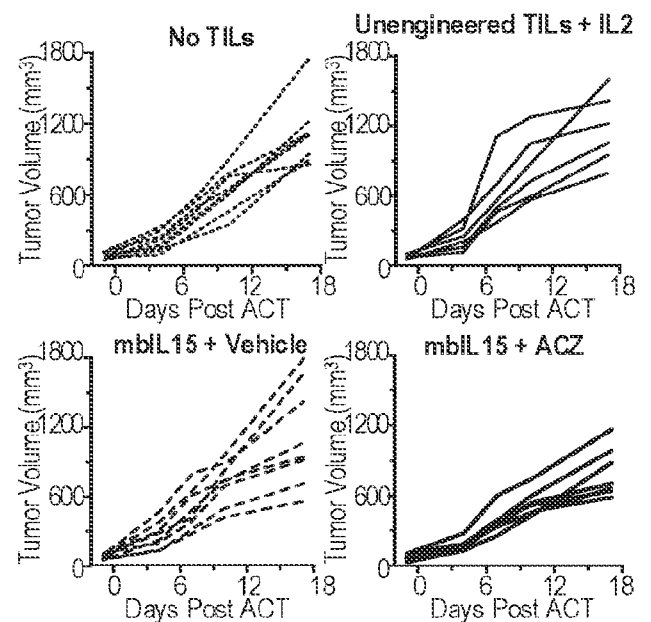


FIG. 14B

Tumor cytotoxicity

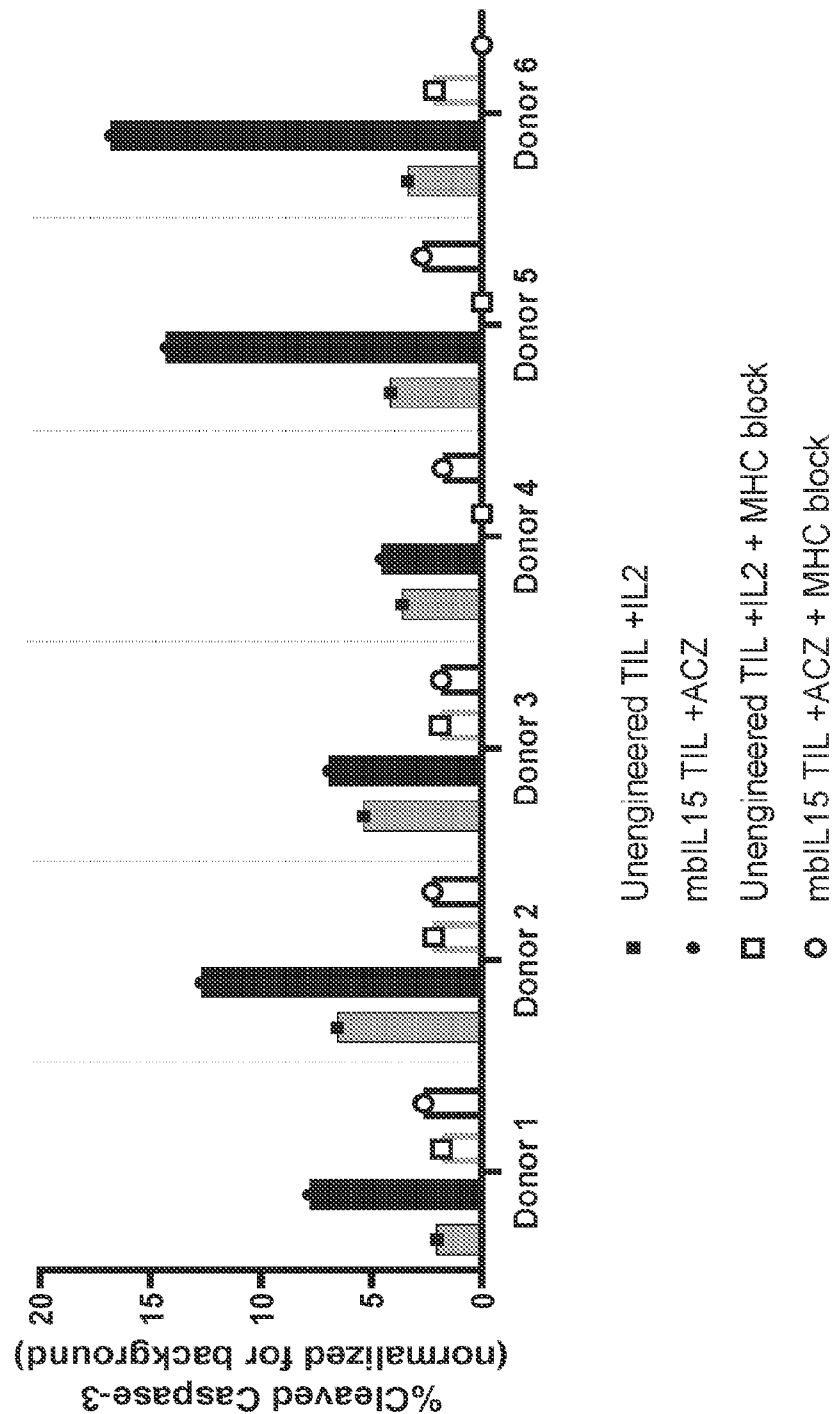


FIG. 15

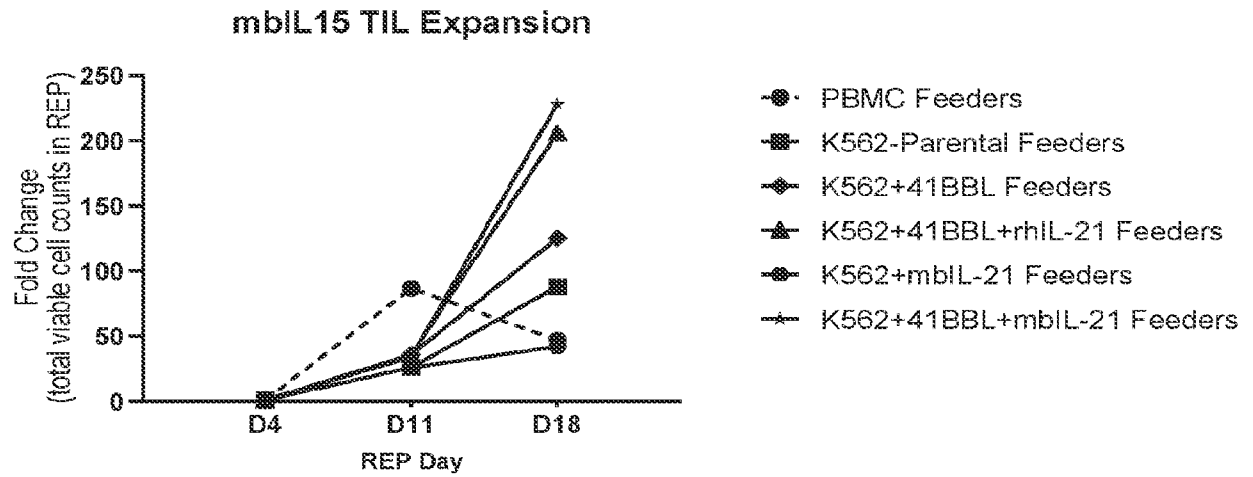


FIG. 16

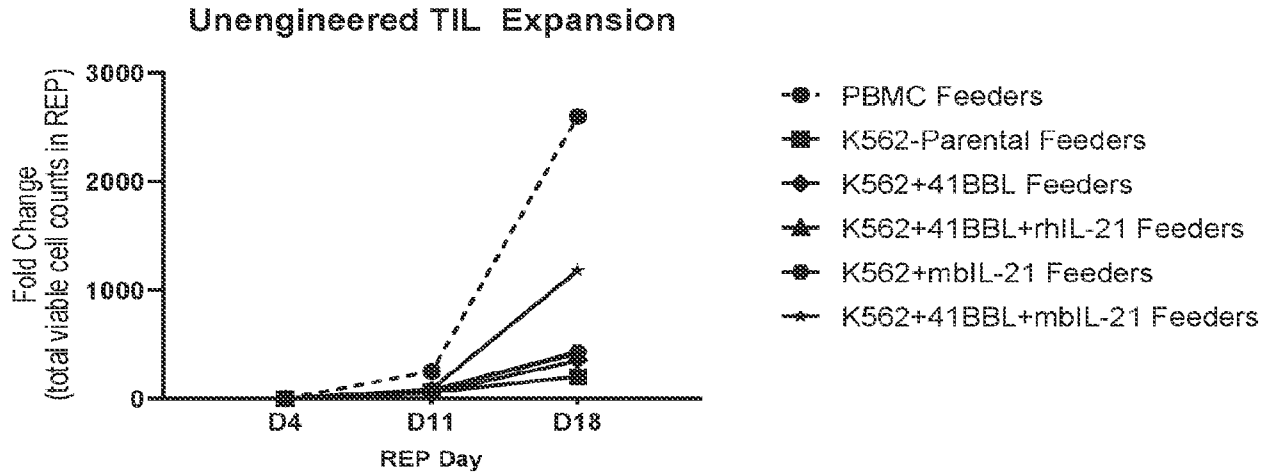


FIG. 17

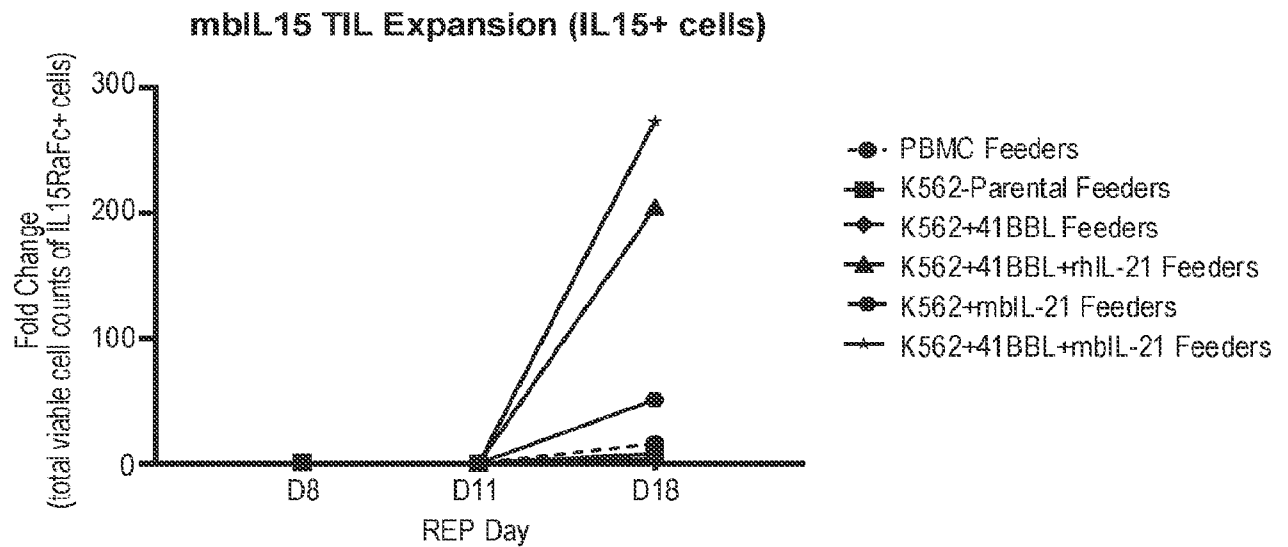


FIG. 18

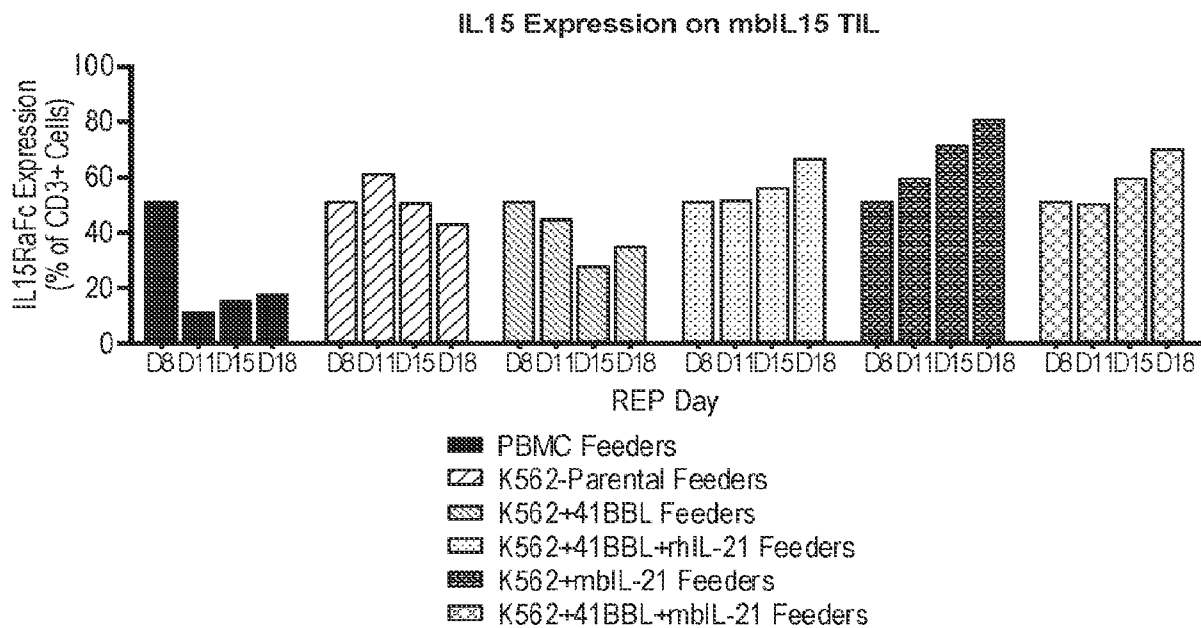


FIG. 19

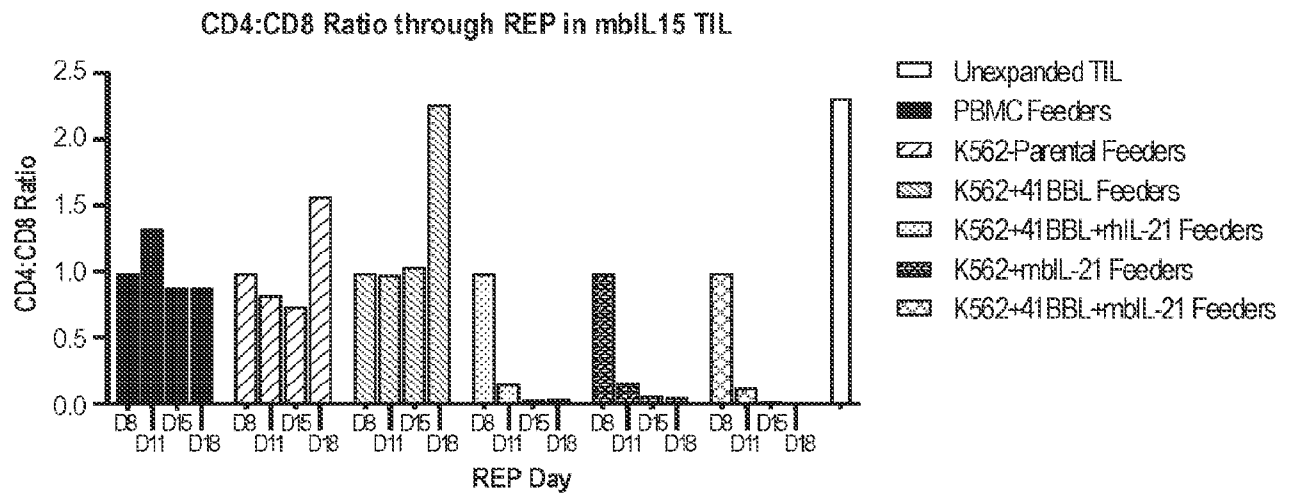


FIG. 20

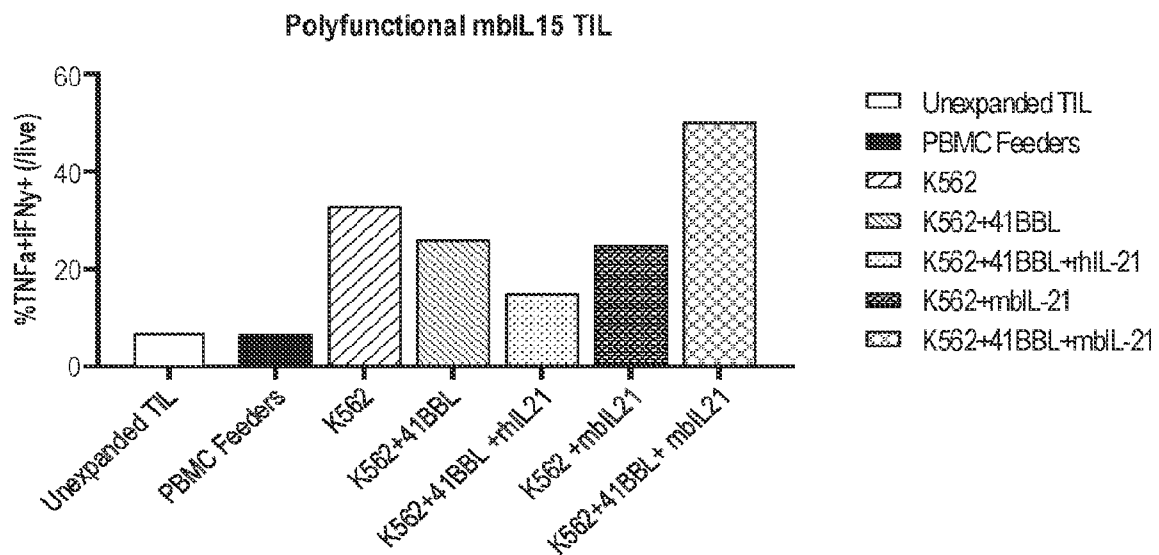


FIG. 21

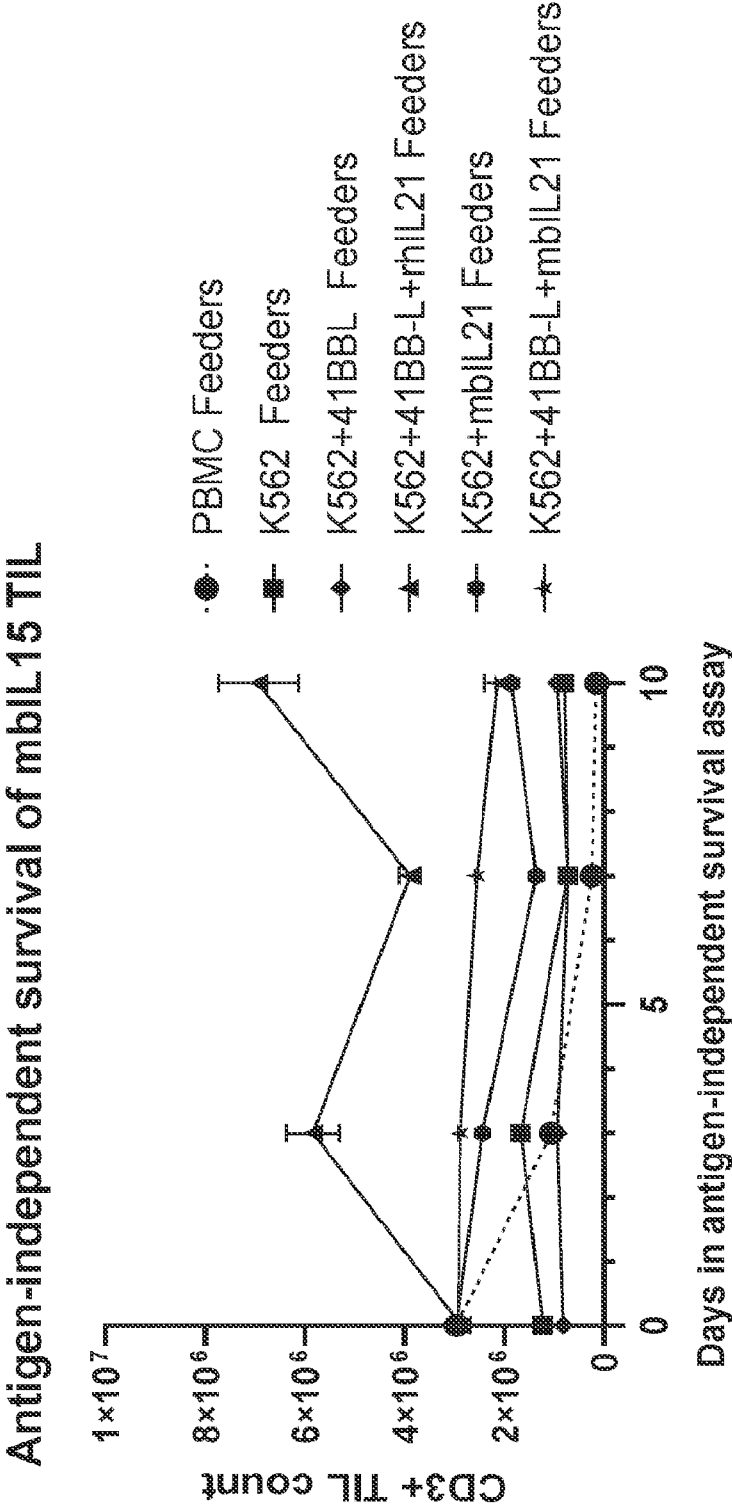


FIG. 22

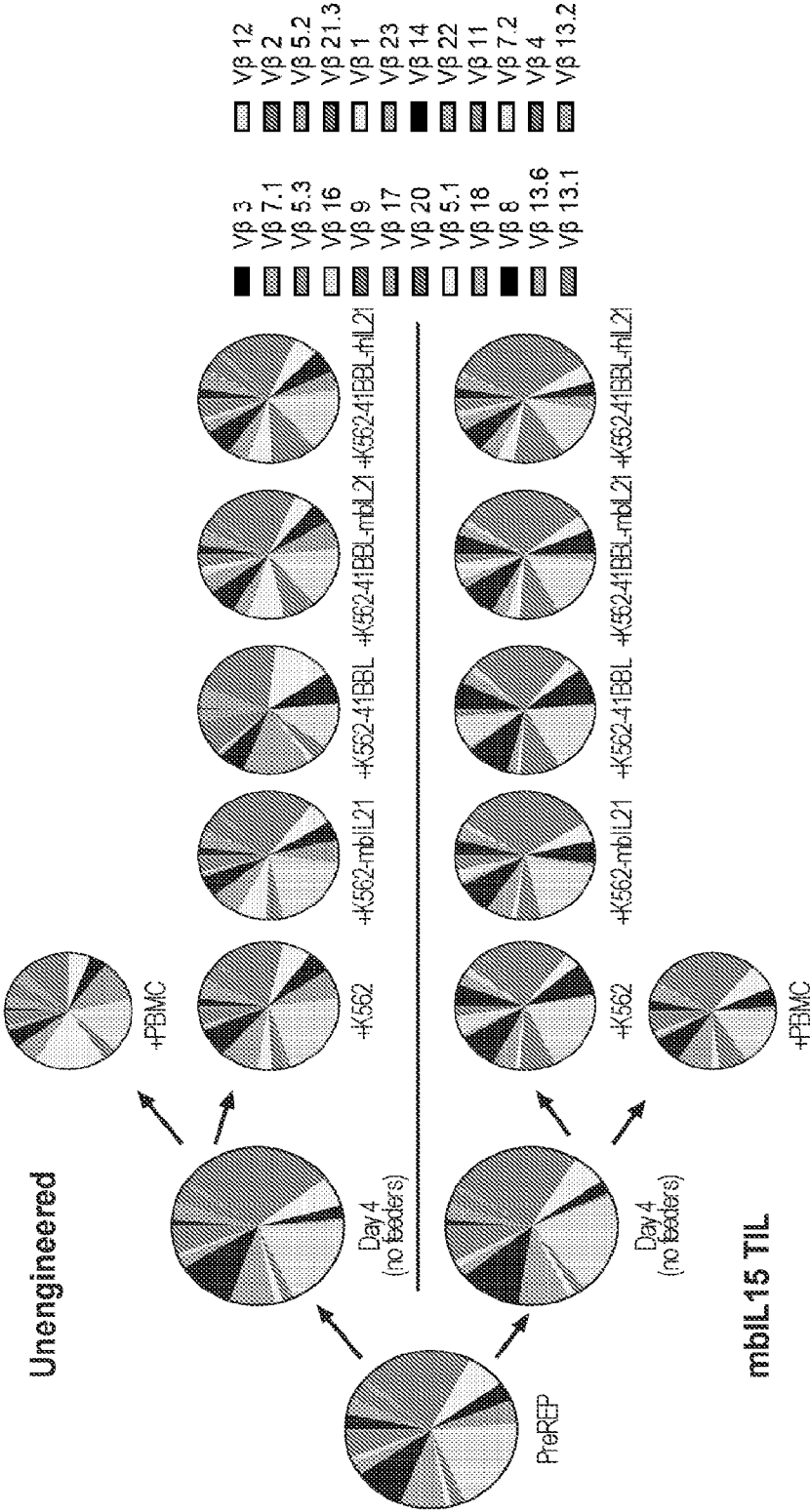


FIG. 23

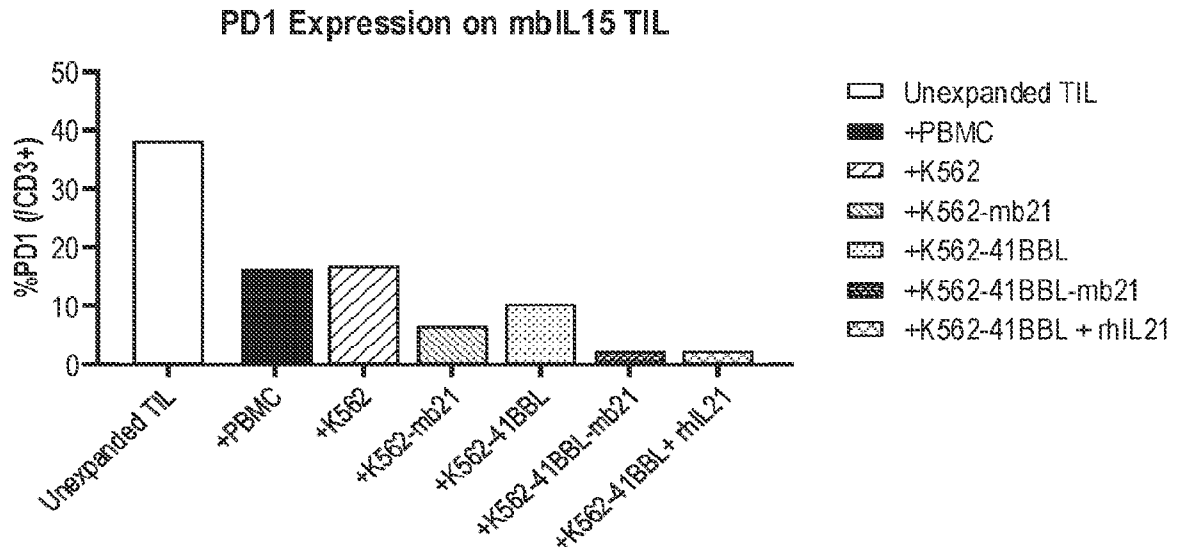


FIG. 24

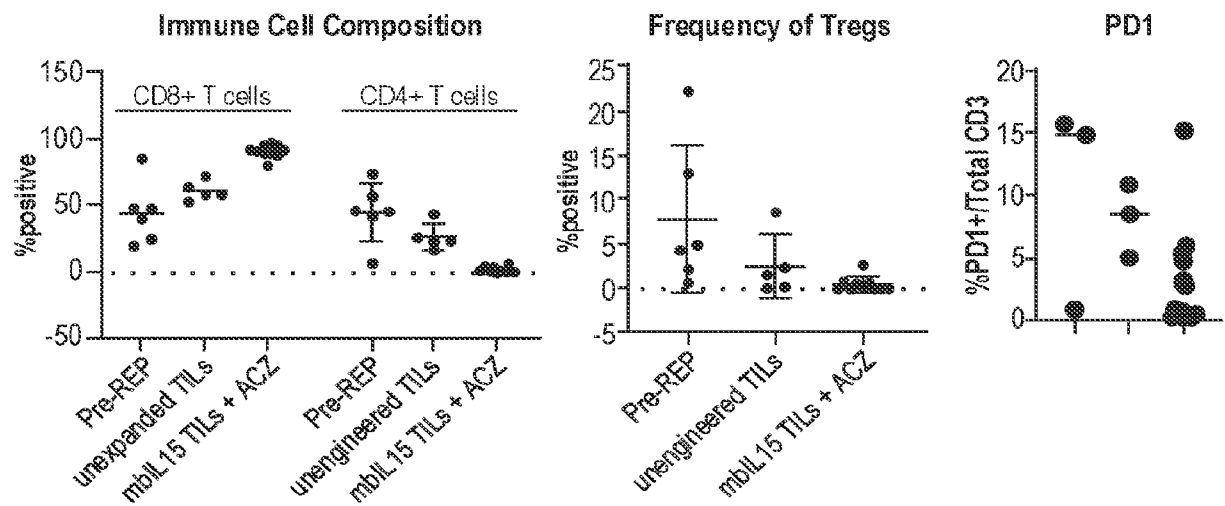
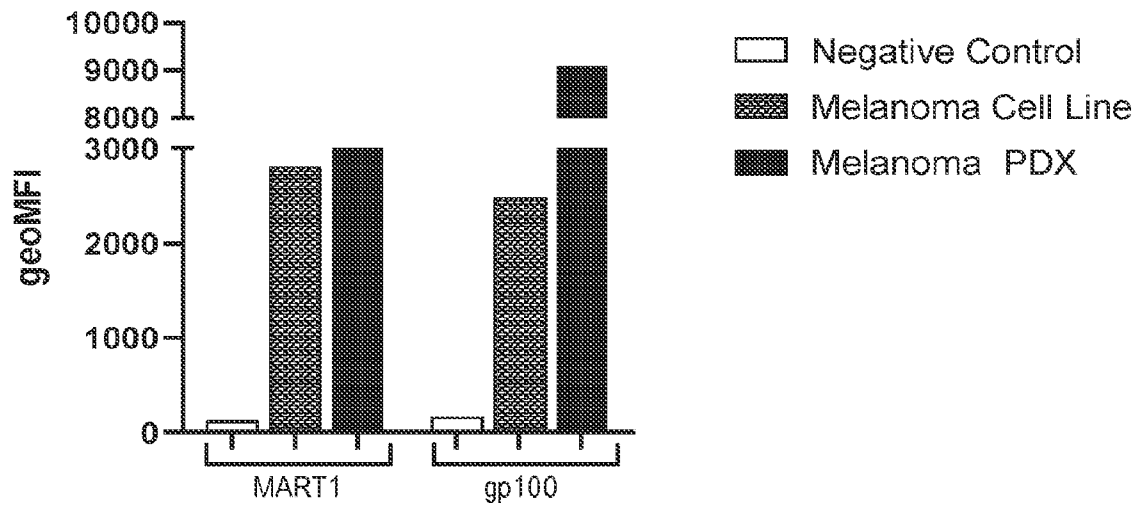
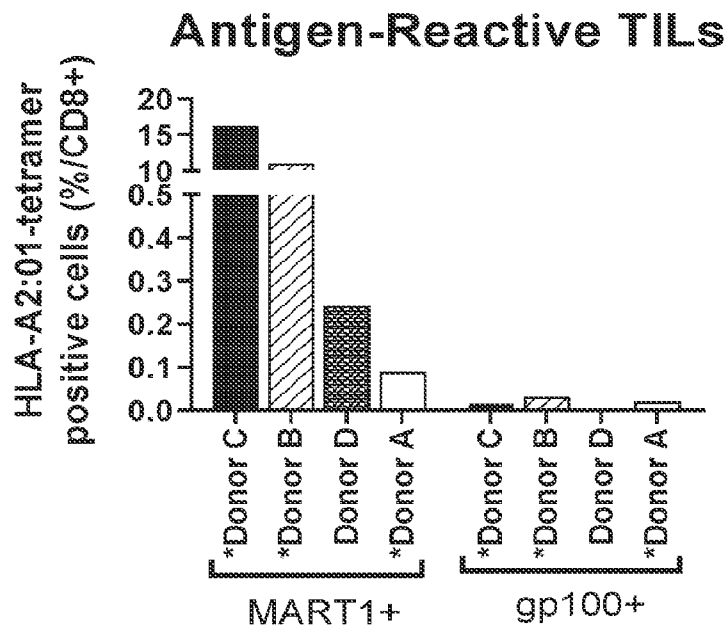


FIG. 25

Melanoma-Associated Antigen Expression

**FIG. 26****FIG. 27**

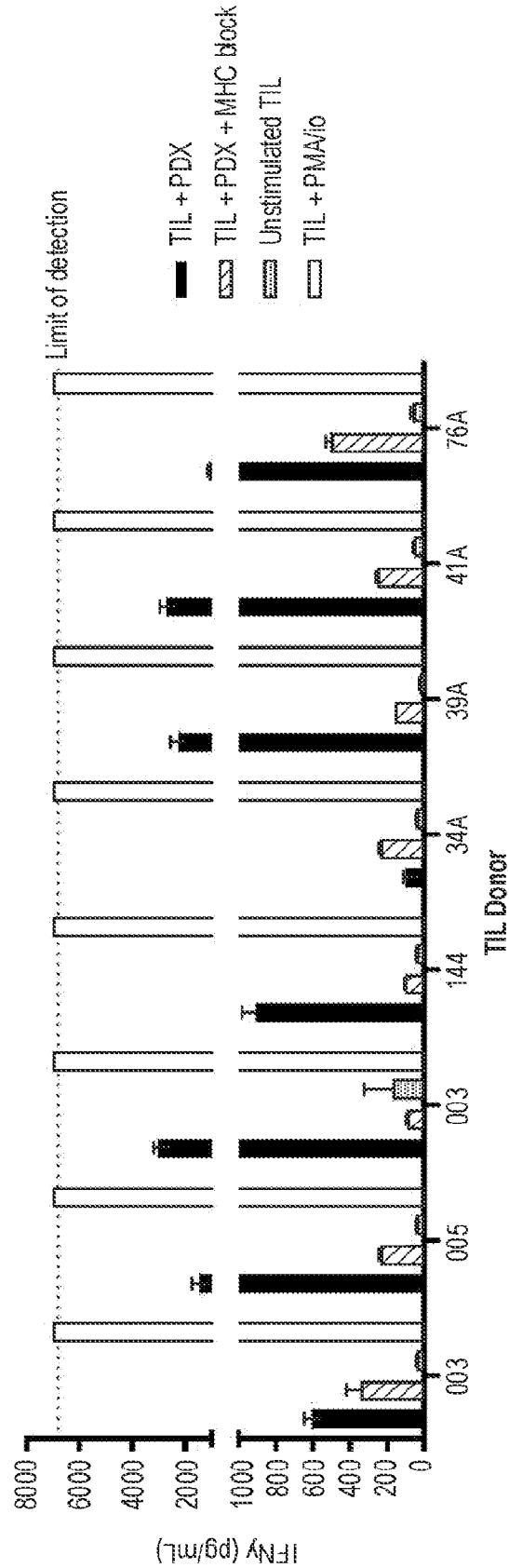


FIG. 28

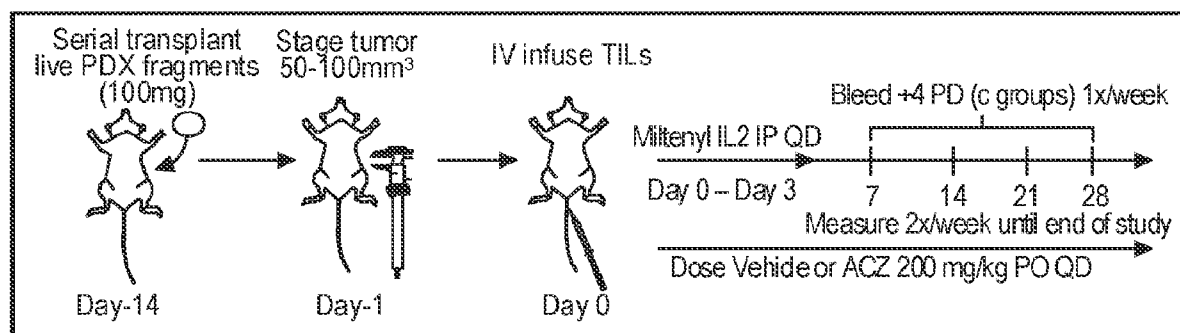


FIG. 29

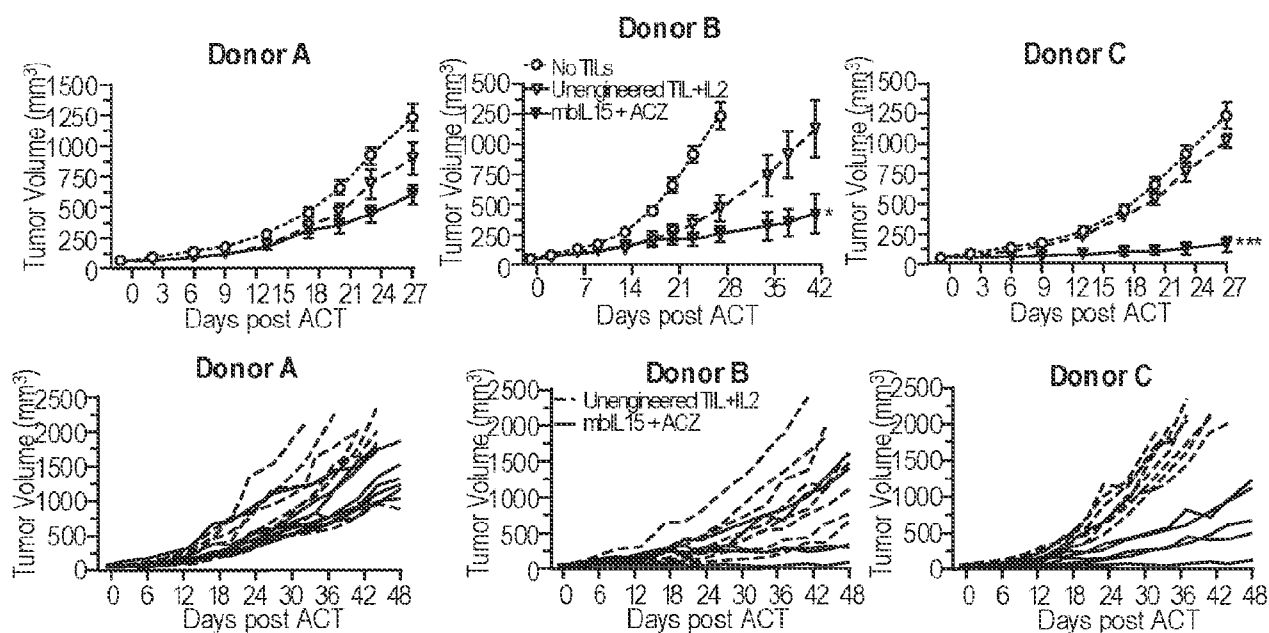


FIG. 30

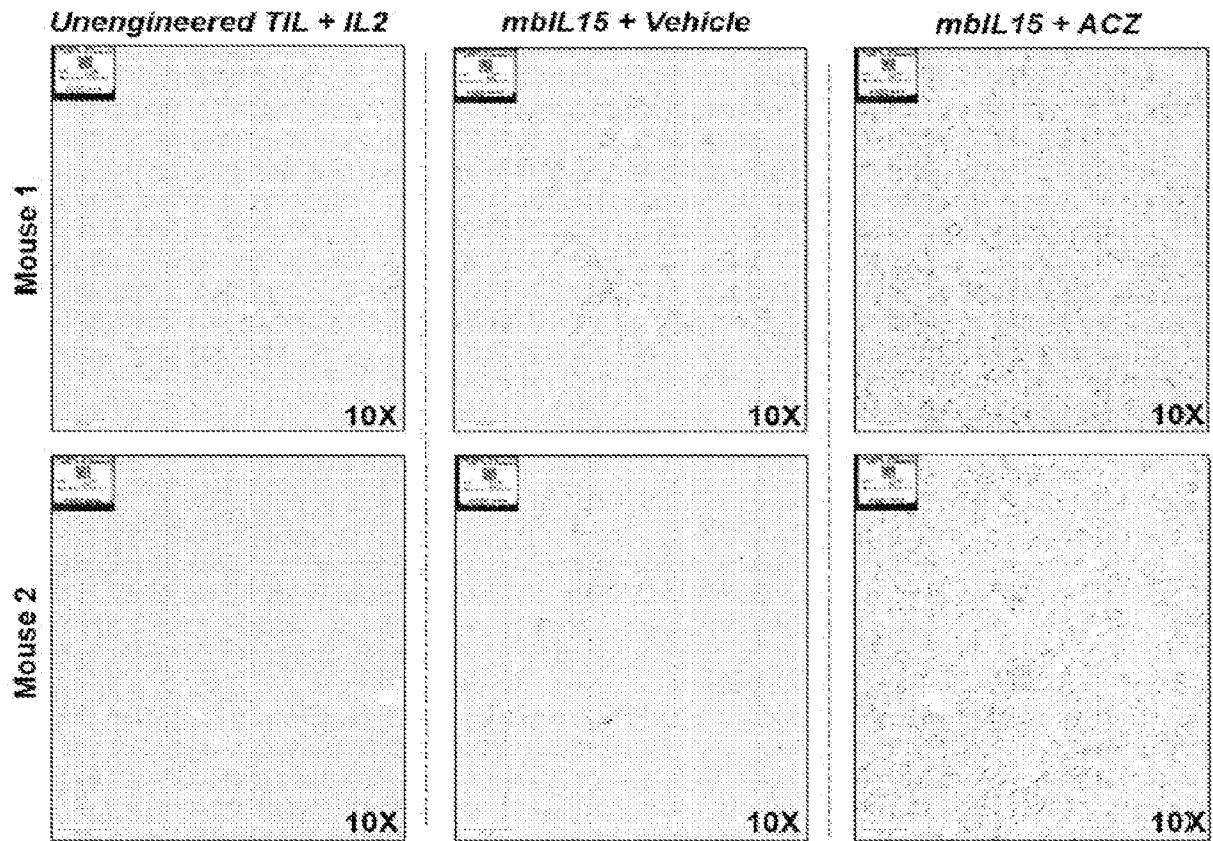


FIG. 31A

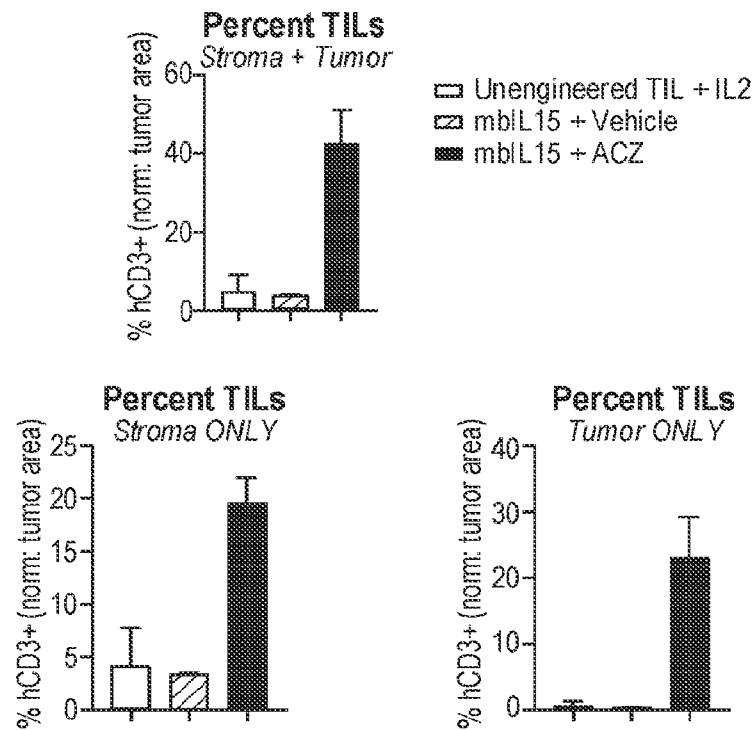


FIG. 31B

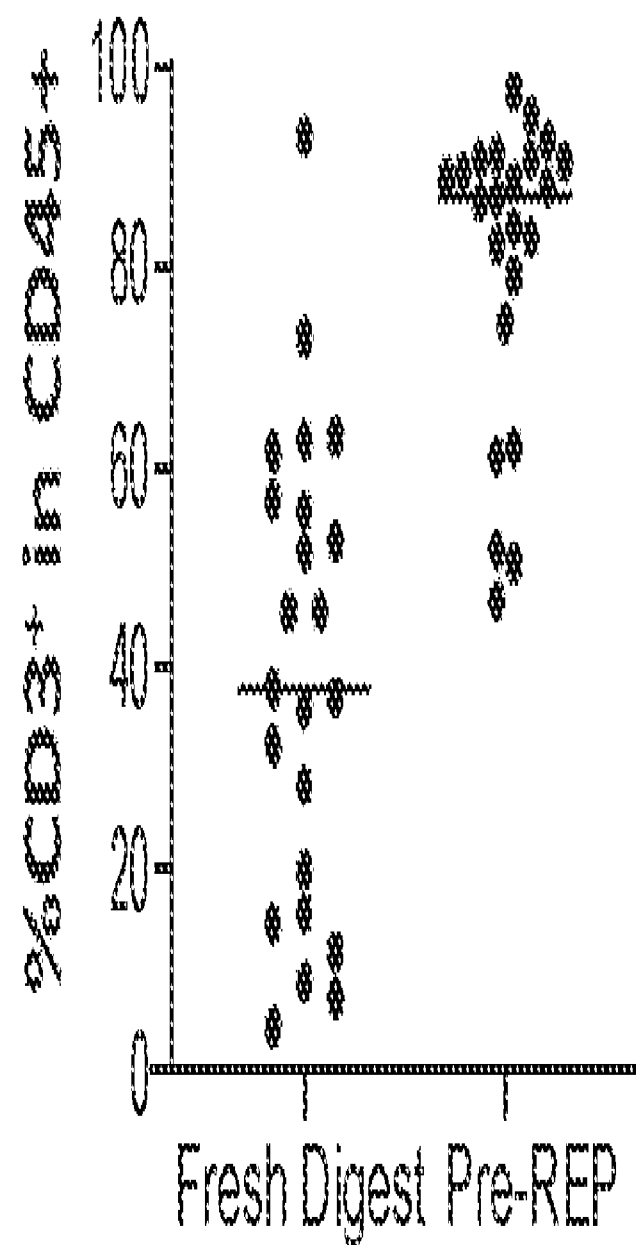
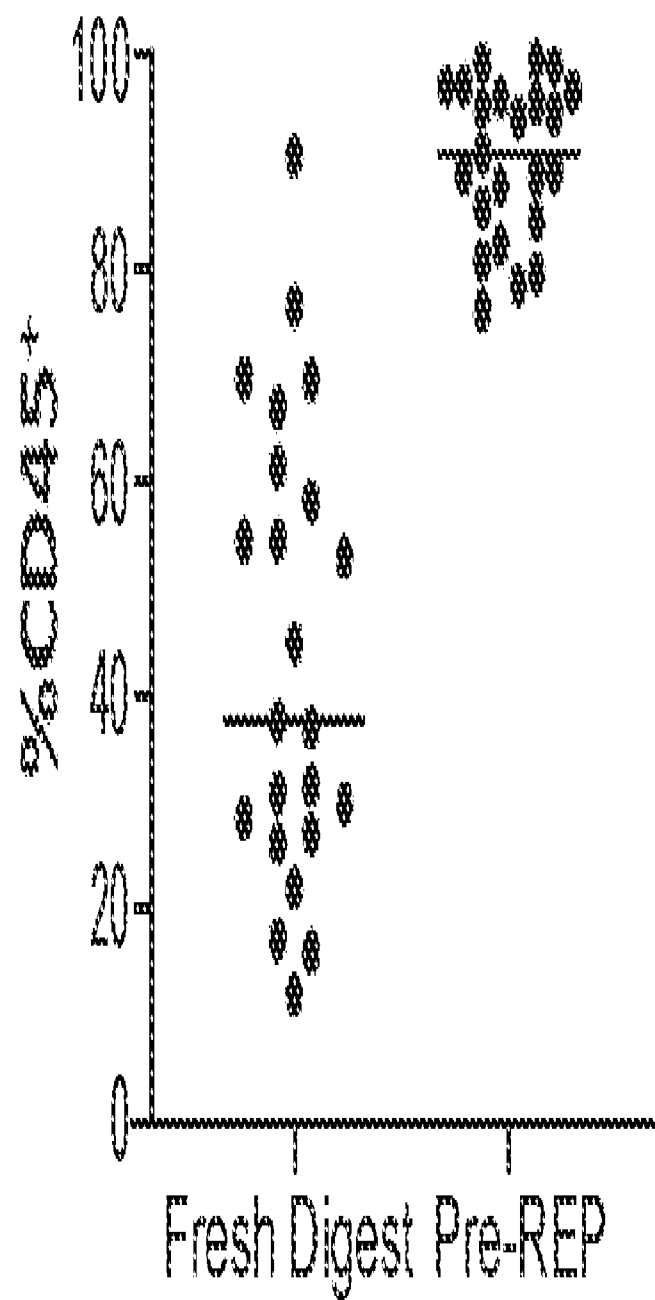


FIG. 1