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(54) Title: ENGINEERED MYELOID PROGENITOR CELLS

(57) Abstract: Disclosed herein are populations of engineered cells comprising a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the cells. The population of engineered cells can be a population of engineered immune cells (e.g., myeloid progenitor cells), or a population of engineered stem cells. Pharmaceutical compositions, kits, and devices comprising the populations of engineered cells are also provided. Also provided herein are methods of treating a subject having a tumor or stimulating an immune response against a tumor in a subject, comprising administering a population of engineered cells described herein.



ENGINEERED MYELOID PROGENITOR CELLS**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims benefit of U.S. Provisional Application No. 63/510,339, filed on June 26, 2023, the entire contents of which are incorporated herein by reference.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing, which has been submitted by Patent Center. The Sequence Listing titled 213661-015002_PCT_SL.xml, which was created on June 6, 2024 and is 3,411 bytes in size, is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present disclosure relates generally to the field of biotechnology and immunotherapy. More specifically, this disclosure pertains to genetically engineered immune cells for treating tumors.

BACKGROUND

[0004] Cancer is a major public health concern worldwide, causing significant morbidity and mortality. Traditional cancer treatments, such as surgery, radiation therapy, and chemotherapy, are often associated with serious side effects and may not be effective in all cases. In recent years, immunotherapy has emerged as a promising alternative or adjunct to conventional cancer therapies. Immunotherapy harnesses the power of the patient's immune system to recognize and eliminate cancer cells, offering a more targeted and potentially less toxic approach to cancer treatment.

[0005] Modulating a patient's immune system using immunotherapeutic approaches has shown remarkable success against hematological neoplasms and some solid tumors, including metastatic melanoma and colorectal carcinoma. Even so, treatment of solid tumors, including glioblastoma, with immunotherapy approaches has been challenging. For instance, while childhood leukemias have shown remarkable responses to T cell-based therapeutics, the treatment of solid tumors has largely been unsuccessful. The treatment of solid tumors

has largely been hindered by the immunosuppressive microenvironment created by solid tumors, which promotes tumor growth and prevents the localization and functions of cytotoxic immune cells responsible for the elimination of transformed cells. Thus, for success in treating solid tumors, immunotherapeutic approaches need to overcome the influence of the tumor microenvironment.

SUMMARY

[0006] This disclosure relates generally to populations of engineered cells, including engineered immune cells or engineered myeloid progenitor cells, that can influence a tumor microenvironment to treat certain tumors, including glioblastoma. The disclosed populations of engineered cells possess the remarkable ability to remodel the tumor microenvironment (TME) into a supportive environment that facilitates cytotoxic immune function and enables effective immunotherapy treatment in brain and other solid tumors. This remodeling effect is achieved through the stable secretion of one or more cytokines, *e.g.*, IL-12. Importantly, the engineered immune cells are designed to secrete IL-12 at levels that effectively suppress tumor growth while ensuring the safety profile associated with a safe treatment for the subject. In some embodiments, the populations of engineered immune cells are genetically modified by the integration of a heterologous nucleic acid encoding the one or more cytokines downstream of a promoter of an endogenous gene. In some embodiments, the endogenous gene includes a sustained transgene expression locus (STEL), for example, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

[0007] In one aspect, this disclosure provides a population of engineered immune cells that secrete interleukin-12 (IL-12) at a concentration of between 1.0 and 1,000 ng/ml, as measured by homogeneous time resolved fluorescence (HTRF), when said cells are cultured at a density of 1 million cells per milliliter for 24 hours. In some embodiments, the population of engineered immune cells secretes IL-12 at a concentration of 500 ng/ml when said cells are cultured at a density of 1 million cells per milliliter for 24 hours.

[0008] In some embodiments, the population of engineered immune cells are genetically modified to comprise a heterologous nucleic acid encoding IL-12.

[0009] In some embodiments, expression of the heterologous nucleic acid is driven by a promoter sequence of an endogenous gene. In some embodiments, the endogenous gene comprises a sustained transgene expression locus (STEL).

[0010] In some embodiments, the endogenous gene comprises Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

[0011] In some embodiments, the population of immune cells comprises one or more of hematopoietic stem cells, myeloid progenitor cells, microglia precursor cells, microglial cells, dendritic cells, T cells, B cells, natural killer cells, or macrophages. In some embodiments, the population of immune cells is a population of myeloid progenitor cells. In some embodiments, the myeloid progenitor cells are derived from pluripotent stem cells, for example, induced pluripotent stem cells (iPSCs).

[0012] In some embodiments, the population of engineered immune cells express CD11b, CD14, and CD45. In some embodiments, at least 80 percent of the engineered immune cells express CD11b, CD14, and CD45. In some embodiments, the population of engineered immune cells are positive for one or more of CD11b, CD14, and CD45. In some embodiments, the engineered immune cells are positive for each of CD11b, CD14, and CD45.

[0013] In some embodiments, the population of engineered immune cells express HLADR, CD86, CD80, and CD40.

[0014] In some embodiments, the population of engineered immune cells do not express CD206, CD163, or MerTK. In some embodiments, the population of engineered immune cells do not express CD206, CD163, and MerTK.

[0015] In some embodiments, the population of immune cells are able to present tumor antigens to other immune cells capable of recognizing antigens. In some embodiments, each cell within the population of the immune cells comprises a major histocompatibility complex (MHC) molecule on the cell surface, and wherein said MHC molecule binds to and presents the tumor antigens to the other immune cells. In some embodiments, antigens may comprise peptide antigens that bind to MHC class I molecules. In some embodiments, antigens may comprise lipid antigens presented by non-classical molecules. In some embodiments, the MHC molecule comprises an MHC class I molecule or an MHC class II molecule.

[0016] In some embodiments, each cell of the population of immune cells comprises a co-stimulatory molecule on the cell surface, and wherein said co-stimulatory molecule enhances an immune response by interacting with immune cells. In some embodiments, the immune response is a CD8⁺ T cell-mediated immune response.

[0017] In some embodiments, the co-stimulatory molecule comprises CD80, CD86, or CD40. In some embodiments, each of the immune cells comprises one or more genetic alterations that results in increased expression of MHC molecule on the cell surface, thereby increasing the capability of the immune cells to present the tumor antigens to T cells.

[0018] In some embodiments, each of the immune cells comprises a targeting moiety on the cell surface. In some embodiments, the targeting moiety comprises an exogenously expressed chimeric or naturally occurring protein capable of binding to an antigen to elicit activation of an immune cell. In some embodiments, the targeting moiety comprises a chimeric antigen receptor. In some embodiments, the targeting moiety comprises a T cell receptor.

[0019] In another aspect, this disclosure provides a method for generating engineered immune cells, including introducing a heterologous nucleic acid encoding IL-12 into a stem cell using a CRISPR/Cas system; and deriving the immune cells from the stem cell, thereby producing the population of engineered immune cells.

[0020] In some embodiments, the stem cell comprises an induced pluripotent stem cell.

[0021] In some embodiments, the heterologous nucleic acid encoding IL-12 is integrated into a sustained transgene expression locus (STEL). In some embodiments, the STEL loci comprises a GAPDH gene. In other embodiments, the heterologous nucleic acid encoding IL-12 is integrated into a sustained transcriptionally active payload region (STAPLR). In some embodiments, the heterologous nucleic acid encoding IL-12 is integrated into the STAPLR, the heterologous nucleic acid further including an exogenous gene promoter.

[0022] In another aspect, this disclosure provides a pharmaceutical composition for treating a tumor, the composition comprising: a population of engineered immune cells as described herein; and further including a pharmaceutical acceptable carrier, carrier, or dilutant.

[0023] In another aspect, this disclosure provides a method of stimulating an immune response against a tumor, the method comprising: administering the population of engineered immune cells as described herein; and allowing the population of engineered immune cells to secrete IL-12, thereby stimulating a CD8⁺ T cell-mediated response against the tumor. In some embodiments, the tumor is a glioblastoma.

[0024] In some embodiments, the administering comprises direct injection of the population of cells into the site of the tumor. In some embodiments, the population of cells stimulates an immune response against the tumor in the subject.

[0025] In some embodiments, the immune response is a CD8⁺ T cell-mediated response. In some embodiments, the immune response comprises secretion of pro-inflammatory cytokines by T regs. In some embodiments, the pro-inflammatory cytokines comprise IFN γ , TNF α , or both IFN γ and TNF α .

[0026] In some embodiments, the method further comprises administering an additional anti-cancer therapy to the subject. In some embodiments, additional anti-cancer therapy comprises a CD28 agonist. In some embodiments, the additional anti-cancer therapy does not comprise a CD28 agonist. In some embodiments, the CD28 agonist is an anti-CD28 antibody.

[0027] In some embodiments, the subject is a human. In another aspect, this disclosure provides a kit comprising a dosage form suitable for administration to a subject comprising the engineered immune cells described herein, and instructional material for the use of said dosage form.

[0028] In another aspect, this disclosure provides a kit comprising a dosage form suitable for administration to a subject comprising a pharmaceutical composition described herein, and instructional material for the use of said dosage form.

[0029] In another aspect, this disclosure provides a device for administering a therapy to a subject comprising a pharmaceutical composition or population of engineered immune cells as described here.

[0030] In another aspect, this disclosure provides an engineered cell comprising a heterologous nucleic acid encoding a cytokine, or a fragment thereof, wherein expression of

the heterologous nucleic acid is driven by a promoter of an endogenous gene. In some embodiments, the endogenous gene comprises a STEL. In some embodiments, the endogenous gene comprises GAPDH. In some embodiments, the cytokine or fragment thereof comprises IL-12. In some embodiments, the engineered cell comprises a stem cell or an immune cell. In some embodiments, the immune cell comprises a myeloid progenitor cell.

[0031] In another aspect, this disclosure further provides a population of cells derived from an engineered cell described herein, wherein the population of cells express the cytokine at a concentration of between 1.0 and 1,000 ng/ml, as measured by homogeneous time resolved fluorescence (HTRF), when said cells are cultured at a density of 1 million cells per milliliter for 24 hours. In some embodiments, the population of cells secrete the cytokine at a concentration below 500 ng/ml, as measured by HTRF, when said cells are cultured at a density of 1 million cells per milliliter for 24 hours.

BRIEF DESCRIPTION OF DRAWINGS

[0032] **FIG. 1** illustrates an exemplary cell differentiation scheme employed to generate myeloid progenitor cells from induced pluripotent stem cells (iPSCs). As detailed here, iPSCs were differentiated into myeloid progenitor cells within a span of 30 to 45 days following induction. The progenitor cells underwent a subsequent maturation process, after which they were subjected to cryopreservation to maintain viability during storage.

[0033] **FIG. 2** is an illustration of the cloning strategy used to integrate IL-12 transgenes into the GAPDH STEL site. The IL-12 transgenes were integrated into the GAPDH STEL site by using a 2A sequence replacing the GAPDH stop codon to integrate the IL-12 transgene in-frame to the endogenous GAPDH sequence before the STOP codon at the 3'UTR. The IL-12 transgenes were integrated using CRISPR-based gene editing systems as described below.

[0034] **FIGS. 3A-3D** show exemplary FACS data collected to characterize iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12. **FIGS. 3A and 3B** show genetically engineered cells express well-established myeloid progenitor cell markers, CD45+, CD11b+, and CD14+. **FIG. 3C** shows an exemplary FACS profile that confirm the

myeloid progenitor cells do not express neutrophil marker CD66b. **FIG. 3D** is an exemplary FACS profile showing the cells do not express proliferation markers Ki67 and pHH3.

[0035] **FIG. 4** shows exemplary experimental results confirming iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12 upregulate M1 markers, *i.e.*, HLADR, and CD86. The results are bar graphs based on FACS data collected from myeloid progenitor cell lines that were co-cultured with T cells. These data show that engineered myeloid progenitor cells (CD19t-IL-12, IL-12 biallelic, and IL-12 monoallelic – as indicated on x-axis), but not “control cells” (*i.e.*, non-edited myeloid progenitor cells), upregulated M1 markers when co-cultured in the presence of T cells.

[0036] **FIG. 5** shows exemplary experimental results demonstrating M2 markers (CD206, CD163, and MerTK) were downregulated by the genetically engineered myeloid progenitor cells after co-culture with T cells. The results are bar graphs based on FACS data collected from myeloid progenitor cell populations co-cultured with T cells. These data show that the engineered myeloid progenitor cells (CD19t-IL-12, IL-12 biallelic, and IL-12 monoallelic – as indicated on x-axis), but not “control cells” (*i.e.*, non-edited myeloid progenitor cells), downregulated genes that are typically present in tumor-associated macrophages and M2 macrophages.

[0037] **FIG. 6** shows exemplary experimental results of cytokine profiles for iPSC derived myeloid progenitor cells genetically engineered to secrete IL-12 and control cells (*i.e.*, non-edited myeloid progenitor cells). The results are bar graphs based on flow-cytometry experiments performed to detect cytokines of interest. These data show a side-by-side comparison of cytokine secretion profiles for two genetically engineered myeloid progenitor cell lines (CD19t-IL-12, and IL-12 monoallelic) and control cells (*i.e.*, non-edited myeloid progenitor cells). The cytokines assayed included IL-4, IL-2, IP-10, IL-1 beta, TNF-alpha, MCP-1, IL-17A, IL-6, IL-10, IFN-gamma, IL-12, IL-8, and TGF-beta 1.

[0038] **FIG. 7** provides exemplary experimental results showing IL-12 output of iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12. In particular, these exemplary data show rates of IL-12 production of control cells (non-edited myeloid progenitor cells) and myeloid progenitor cells generated from iPSCs genetically engineered to secrete IL-12 according to three different engineering strategies. The rate of IL-12 produced was measured using homogeneous time resolved fluorescence (HTRF). The amount

of IL-12 produced is identified on the y-axis in nanograms per milliliter per one million cells per twenty-four hours (IL-12 ng/mL/1M/24hr). The control and genetically engineered myeloid progenitor cell lines are identified on the x-axis.

[0039] **FIG. 8** shows exemplary experimental results demonstrating iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12 effectively kill tumor cells and inhibit tumor cell growth in vitro. **FIG. 8** is exemplary data taken from cell killing assays that compare varying quantities of two genetically engineered myeloid progenitor cell lines—one line with CD19t-IL-12 integration, the other with monoallelic, IL-12 integration—and control cells (non-edited myeloid progenitors). The y-axis shows the number of apoptotic U251 cells. The x-axis identifies the cells (and cell populations) present in the corresponding wells.

[0040] **FIGS. 9A and 9B** show exemplary experimental results demonstrating the ability of genetically engineered myeloid progenitor cells to kill tumor cells, thus leading to increased tumor cell death. These figures show bar graphs quantifying the growth of U251 tumor cells in co-culture with CD8 T cells and varying quantities of control cells (**FIG. 9A**), as compared with genetically engineered myeloid progenitor cell lines: monoallelic IL-12 engineered myeloid progenitor cells (**FIG. 9B**). The x-axis represents cell numbers for each cell type, while the y-axis corresponds to U251 tumor cell growth. Measurements were taken after a 5-day co-culture period.

[0041] **FIG. 10** illustrates an exemplary workflow that was used to evaluate the impact of M2 polarization on CD8 T cell activity mediated by genetically modified myeloid progenitor cells. Myeloid progenitor cells derived from iPSCs genetically engineered to secrete IL-12 and control cells (myeloid progenitor cells derived from non-edited iPSCs) were cultured, separately, under conditions that simulate the tumor microenvironment. This stimulation involved culturing cell populations in the presence of TGF-beta and IL-10 to induce M2 skewing. In parallel, CD8 T cells were separated from donor PBMCs using a commercially available CD8 T cell isolation kit. Subsequently, the isolated CD8 T cells were co-cultured with M2-skewed myeloid progenitor cells. Following this co-culture, the supernatants were evaluated by flow cytometry-based assays to measure the levels IL-12, IFN-gamma, TNF-alpha, IL-2, Perforin, Granzyme A, Granzyme B, and Granulysin.

[0042] **FIGS. 11A and 11B** are exemplary experimental results that show myeloid progenitor cells rescue IFN-gamma secretion from CD8 T cells despite M2 polarization. Specifically, **FIG. 11A** shows the amounts of IFN-gamma (y-axis) that was detected by flow cytometry following the co-culture of T cells with varying quantities of M2 polarized myeloid progenitor cells (indicated on x-axis). This data demonstrate that the secretion of IFN-gamma by T cells is rapidly elevated by the addition of genetically engineered myeloid progenitor cells despite M2 polarization. **FIG. 11B** is exemplary data showing the amounts of IL-12 (y axis) that was detected by varying quantities of M2 polarized myeloid progenitor cells (identified along x-axis).

[0043] **FIGS. 12A-12G** are exemplary experimental results showing myeloid progenitor cells genetically engineered to secrete IL-12 activate CD8 T cell cytolytic activity despite M2 polarization. More particularly, **FIG. 12A** shows the amount of IL-12 (indicated on y-axis) that was detected from the cell populations indicated on the x-axis. **FIGS. 12B-12G** show the amounts of effector CD8 T cell cytokines and cytolytic enzymes (TNF-alpha, IL-2, Perforin, Granzyme A, Granzyme B, and Granulysin - as indicated) that was detected from corresponding cell populations.

[0044] **FIG. 13** provides exemplary data showing the significant tumor suppressive capabilities of genetically engineered myeloid progenitor cells, *in vivo*. Specifically, **FIG. 13** shows line graphs of tumor volumes (indicated on y-axis) that were detected over time (indicated on x-axis) from mice across different treatment groups. The upper panel shows all mice in the study and the lower panels shows individual mice per treatment group. These exemplary data demonstrate the anti-tumor efficacy of genetically engineered myeloid progenitor cells.

[0045] **FIGS. 14A and 14B** show exemplary experimental results further demonstrating the *in vivo* therapeutic efficacy of genetically engineered myeloid progenitor cells. **FIG. 14A** shows exemplary data that was obtained by collecting tumor measurements from pre-clinical animal models of glioblastoma (GBM) that had been subjected to the indicated treatments (shown on the x-axis). Surprisingly, these exemplary data clearly demonstrate a significantly lower tumor growth inhibition ratio (y-axis) when using myeloid progenitor cells genetically engineered to secrete IL-12 than control non-engineered cells or anti-PD1 monoclonal antibody benchmark. **FIG 14B** shows representative images of the tumors which were

harvested from the pre-clinical mouse models at humane end points. The tumors were cleared of surrounding fat and muscle tissue to ensure accurate size measurements.

[0046] **FIG. 15** shows IL-12 levels detected in the serum of pre-clinical animal models over a 20-day period. Serum samples were collected from the animal models at specific time points as indicated, and IL-12 secretion was analyzed using bead-based Luminex approaches.

[0047] **FIGS 16A-16C** provide exemplary results showing serum cytokine levels associated with cytokine release syndrome (CRS), as compared to treatment with an anti-PD-1 antibody. The evaluation was performed on the intratumoral (IT) treatment with genetically engineered myeloid progenitor cells (CD19t-IL-12) in comparison to the benchmark monoclonal antibody (mAb) treatment anti-human PD-1 (aPD1) administered intraperitoneally (IP). The data were obtained from humanized mice, where human flank tumors were established, followed by the transfer of human peripheral blood mononuclear cells (huPBMCs). Subsequently, the mice received either the myeloid progenitor cell treatment or aPD1 treatment. The evaluation of CRS was performed by analyzing the serum cytokine levels via Luminex at day 14 post treatment. **FIG. 16A** shows levels of IL-6 (pg/ml) on the Y axis compared across treatment groups: untreated, vehicle, control cells, genetically engineered myeloid progenitor cells (CD19t-IL-12), and aPD1. **FIG. 16B** shows levels of IL-8 (pg/ml) on the Y axis compared across treatment groups: untreated, vehicle, control cells, genetically engineered myeloid progenitor cells (CD19t-IL-12), and aPD1. **FIG. 16C** shows levels of IL1-beta (pg/ml) on the Y axis compared across treatment groups: untreated, vehicle, control cells, genetically engineered myeloid progenitor cells (CD19t-IL-12), and aPD1.

[0048] **FIG. 17A-17D** show exemplary experimental results that demonstrate the remodeling of the tumor microenvironment (TME) towards an anti-tumor cytolytic T lymphocyte (CTL) response following the administration of myeloid progenitor cells. In **FIG. 17A**, experiment data showing the ratios of anti-tumor cytolytic CD8 T cells to pro-tumor immunosuppressive FoxP3+ T cells (Tregs) in the tumor after treatment with myeloid progenitor cells genetically engineered to secrete IL-12 or control cells (non-edited myeloid progenitor cells). **FIG. 17B** demonstrates an infiltration of T cells (CD3+) within the TME with an overall decreased exhaustion phenotype (PD1/TIM3 negative), indicating a shift from immunosuppressive to pro-inflammatory phenotypes. **FIG. 17B** demonstrates an increased frequency of total T cells within the TME, and **FIGS. 17C and 17D** show a decrease in

exhaustion markers (PD1/TIM3) within CD4 and CD8 subsets in the TME. This observation suggests that the shift in T cell frequency is accompanied by a functional change in T cell activation in the TME by *in vivo* intratumoral (IT) treatment with genetically engineered myeloid progenitor cells.

[0049] **FIGS. 18A and 18B** show exemplary experimental results demonstrating that engineered M2 polarized myeloid progenitor cells (indicated as MPCs) can restore both CD8 IFN γ (**FIG. 18A**) and TNF α (**FIG. 18B**) to a level of pro-inflammatory cytokine that is comparable between CD8 T cells stimulated with or without anti-CD28 monoclonal antibody (mAb). **FIGS. 18A and 18B** are bar graphs of exemplary results showing levels of IFN γ (pg/mL) and TNF α (pg/mL), respectively, secreted from CD8 T cells under conditions identified along the X-axis. **FIG. 18A** compares levels of IFN γ secreted from CD8 T cells when the cells are cultured with or without M2 myeloid progenitor cells, and with and without the addition of an anti-CD28 mAb. **FIG. 18B** compares levels of TNF α secreted from CD8 T cells when the cells are cultured with or without M2 myeloid progenitor cells, and with and without the addition of an anti-CD28 mAb.

[0050] **FIGS. 19A and 19B** show exemplary results demonstrating the impact of myeloid progenitor cells (and control cells) on tumor spheroid size (x axis) when cultured with anti-CD3 mAb alone (**FIG. 19A**) or with a combination of anti-CD3 and anti-CD28 mABs (**FIG. 19B**). The dark lines indicate observations from myeloid progenitor cells. The gray lines indicate observations from control cells.

[0051] **FIGS. 20A and 20B** show exemplary results demonstrating myeloid progenitor cells (indicated as MPCs) promote pro-inflammatory cytokine production from Tregs. **FIG. 20A** shows levels of IFN γ secreted from Tregs co-cultured with a certain number of myeloid progenitor cells indicated along the X axis. The Y axis indicates the fold change in IFN γ (as measured in pg/mL) from myeloid progenitor cells in combination with Tregs as compared with Tregs only. **FIG. 20B** shows levels of TNF α secreted from Tregs co-cultured with a certain number of engineered myeloid progenitor cells indicated along the X axis. The Y axis indicates the fold change in TNF α (as measured in pg/mL) from engineered myeloid progenitor cells in combination with Tregs as compared with Tregs only.

DETAILED DESCRIPTION

[0052] Aspects of the present disclosure relate to methods, populations of engineered cells, pharmaceutical compositions, kits, and devices for stimulating an immune response against a tumor in a subject. In particular, the methods, populations of engineered cells, pharmaceutical compositions, kits, and devices for stimulating an immune response against a tumor in a subject include a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that secrete IL-12. In some embodiments, the population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that secrete IL-12 has a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the engineered myeloid progenitor cells as compared to comparable wild-type cells. In some embodiments, the population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) can elicit or enhance an immune response (*e.g.*, an anti-tumor immune response) in a subject.

[0053] The immune response has an important role in cancer, including for the identification and elimination of tumors. For example, transformed cells of tumors can express antigens (*e.g.*, tumor antigens) that are not found on normal cells. To the immune system, these antigens appear foreign, and their presence causes immune cells to attack the transformed tumor cells. The main response of the immune system to tumors is to destroy the abnormal cells using killer T cells, sometimes assisted by helper T cells. Tumor antigens are presented on MHC molecules, which allows cytotoxic T cells and NK cells to recognize the tumor cell as abnormal, as well as the generation of antibodies against the tumor cells allowing for their destruction by the complement system.

[0054] In the embodiments described herein, there are populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) for treatment of a subject having a tumor (*e.g.*, glioblastoma). The population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) can remodel a tumor microenvironment (TME) into a microenvironment that supports cytotoxic immune function and support effective immunotherapy treatment (*e.g.*, CAR-T therapy) in brain and other solid tumors through the stable secretion of one or more cytokines, such as, IL-12.

[0055] Interleukin-12 is a heterodimeric cytokine with multiple biological effects on the immune system. It includes two subunits, p35 and p40, both of which are required for the secretion of the active form of IL-12, p70. Interleukin-12 acts on dendritic cells (DC), leading to increased maturation and antigen presentation, which can allow for the initiation of a T cell response to tumor specific antigens. It also drives the secretion of IL-12 by dendritic cells, creating a positive feedback mechanism to amplify the response. Once a response is initiated, IL-12 plays a fundamental role in directing the immune system in activating a CD4⁺ T cell-mediated and/or a CD8⁺ T cell response. Furthermore, IL-12 can promote the activation of innate immune cells such as macrophages and eosinophils through its induction of other pro-inflammatory cytokines. This activation then leads to IL-12 secretion by these cells and further amplification of both innate and adaptive immune responses.

[0056] The embodiments described herein use novel populations of engineered cells (*e.g.*, population of engineered immune cells or populations of engineered myeloid progenitor cells) a broadly applicable therapy that will elicit or enhance an anti-tumor response in a subject. Such novel populations are based, at least in part, on the surprising and unexpected observation that secretion of specific amounts of IL-12 by engineered cells, such as engineered immune cells or engineered myeloid progenitor cells, activates cytotoxic T cells and orchestrates remodeling of the tumor microenvironment to promote tumor elimination through the enhancement of anti-tumor immune responses. Thus, the population of engineered cells described herein present an improvement in the treatment of solid tumors, such as glioblastoma.

[0057] Furthermore, this disclosure provides populations of engineered cells (*e.g.*, myeloid progenitor cells) that secrete IL-12 at a therapeutic rate that ensures antitumoral efficacy while minimizing the risk of toxicity. As discussed, IL-12 is a potent cytokine with immunomodulatory properties that plays a crucial role in anti-tumor immune responses. However, excessive IL-12 production may lead to adverse effects, including systemic toxicity. To address this challenge, the disclosed populations of engineered cells are genetically modified to achieve a rate of IL-12 secretion that activates an immune response against tumors without reaching toxic concentrations.

[0058] Through extensive research and optimization, the disclosure provides strategies for generating populations of engineered cells that secrete an optimal level of IL-12 secretion. For example, this disclosure describes engineering strategies that result in the production of

engineered cells with IL-12 secretion profiles that maximize therapeutic efficacy while minimizing toxicities associated with IL-12 overexpression. Accordingly, the disclosed engineered cells offer an improved approach for cancer immunotherapy, which harnesses the benefits of IL-12 while addressing the challenges associated with toxicity. The controlled secretion of IL-12 by these cells ensures a therapeutic effect within a safe range, providing a new avenue for enhancing anti-tumor immune responses without compromising patient safety. This innovation opens up opportunities for the development of targeted and personalized treatments, as the precise regulation of IL-12 secretion can be tailored to individual patient needs. Overall, the disclosure of myeloid progenitor cells with optimized IL-12 secretion represents a significant advancement in the field of immunotherapy, which can revolutionize cancer treatment strategies.

[0059] For example, this disclosure provides an engineered cell (*e.g.*, myeloid progenitor cell) that includes a heterologous nucleic acid encoding IL-12 downstream of a promoter of an endogenous gene associated with a sustained transgene expression locus (STEL), such as, GAPDH. The approach of integrating IL-12 behind the GAPDH promoter presents an important breakthrough in immunotherapy. IL-12, a potent cytokine, is pivotal in modulating anti-tumor immune responses. However, its excessive production can lead to systemic toxicity, posing challenges for therapeutic applications. By placing IL-12 behind the GAPDH promoter—an endogenous gene whose consistent expression is important for cellular function—the engineered cells can exploit endogenous expression mechanisms to control IL-12 production. This arrangement ensures that IL-12 secretion, driven by the stable expression of the GAPDH gene, remains within non-toxic levels, yet retains the potency required for antitumoral efficacy. When cultured at certain densities, these cells secrete IL-12 at concentrations that strike an optimal balance between therapeutic impact and safety.

[0060] Furthermore, the integration of IL-12 into stem cells, particularly into a STEL, offers remarkable stability and consistency of cytokine expression through different stages of cellular differentiation. Evaluation of IL-12 secretion from engineered stem cells exhibit a notable consistency with the levels secreted from myeloid progenitor cells. This uniformity in secretion levels across different cell types and developmental stages underscores the advantage of utilizing a STEL (*e.g.*, the GAPDH locus) for cytokine integration; that is, the expression levels remain essentially unaltered following differentiation. The invariable expression of GAPDH, an endogenous gene, across diverse cell types and developmental

stages offers a stable platform for the sustained and predictable expression of IL-12. This constancy is not only beneficial for maintaining therapeutic efficacy and minimizing toxicity but also proves advantageous during the research and development phase, where researchers can evaluate and select preferred clones secreting the desired IL-12 levels right from the induced pluripotent stem cell (iPSC) stage. Thus, the GAPDH locus emerges as a reliable and strategic genomic location for the stable expression of therapeutic genes like IL-12, facilitating both the research process and subsequent therapeutic applications.

[0061] Although the disclosure describes various exemplary alternatives and implementations as provided herein, it should be understood that the various features, aspects, and functionality described in one or more of the individual alternatives are not limited in their applicability to the particular alternative with which they are described. Instead, they can be applied alone or in various combinations to one or more of the other alternatives of the disclosure, whether the alternatives are described or whether the features are presented as a part of the described alternative. The breadth and scope of the present disclosure should not be limited by any exemplary alternatives described or shown herein.

I. Definitions

[0062] The following definitions supplement those in the art and are directed to the present disclosure only. The following definitions are not to be imputed to any related or unrelated case, *e.g.*, to any commonly owned patent or patent application. Although some methods and materials similar or equivalent to those described herein can be used to practice features of the disclosure, some preferred materials and methods are described herein. Accordingly, the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0063] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[0064] It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such may vary. The

terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention, which is defined solely by the claims.

[0065] As used herein, the articles “a,” “an,” and “the” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

[0066] The use of the alternative (*e.g.*, “or”) should be understood to mean either one, both, or any combination thereof of the alternatives.

[0067] As used herein, the term “about” or “approximately” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In some instances, the term “about” or “approximately” refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length $\pm$ 15%, $\pm$ 10%, $\pm$ 9%, $\pm$ 8%, $\pm$ 7%, $\pm$ 6%, $\pm$ 5%, $\pm$ 4%, $\pm$ 3%, $\pm$ 2%, or $\pm$ 1% of a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[0068] As used herein, the terms “administration,” “administering” and variants thereof refer to the introduction of a composition or therapeutic agent (*e.g.*, a population of cells) into a subject. Administration includes concurrent and sequential introduction of the composition or therapeutic agent. Administration of the composition or therapeutic agent (*e.g.*, a population of cells) into a subject is by any suitable route, including orally, pulmonarily, intranasally, parenterally (intravenously, intramuscularly, intraperitoneally, or subcutaneously), rectally, intralymphatically, or topically. A suitable route of administration allows the composition or the agent to perform its intended function. Administration also includes self-administration and the administration by another. The administration can also be performed systemic, or it can be local. For instance, a composition or therapeutic agent (*e.g.*, a population of cells) can be administered locally, *e.g.*, by local injection into a tissue.

[0069] As used herein, the term “and/or” should be understood to mean either one, or both of, or any combination of the alternatives.

[0070] As used herein, the term “antibody” refers to an immunoglobulin molecule which specifically binds with an antigen (*e.g.*, a cell surface antigen). Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)₂, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, N.Y.; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

[0071] As used herein, the term “anti-cancer therapy” refers to any therapy used for treating a cancer in a subject. Exemplary anti-cancer therapies include, but are not limited to, hormone blocking therapy, chemotherapy, small molecule drugs, kinase inhibitor, therapeutic antibodies or binding fragments thereof, cell therapies, radiation, and surgery.

[0072] As used herein, the term “antigen” refers to a molecule that provokes an immune response. This immune response can involve either antibody production, or the activation of specific immunologically-competent cells (*e.g.*, T cells), or both. Antigens can be any type of molecule including, for example, peptides, haptens, simple intermediary metabolites, sugars (*e.g.*, oligosaccharides), lipids, and hormones as well as macromolecules such as complex carbohydrates (*e.g.*, polysaccharides), phospholipids, and proteins. For example, the term antigen can refer to a substance that induces an immune response. Common categories of antigens include, but are not limited to, viral antigens, bacterial antigens, fungal antigens, protozoan and other parasitic antigens, tumor antigens, antigens involved in autoimmune disease, allergy and graft rejection, toxins, and other miscellaneous antigens.

[0073] As used herein, the term “antigen-presenting cell” or “APC” refers to a cell that can process and display foreign antigens (*e.g.*, tumor antigens) in association with major histocompatibility complex (MHC) molecules on its surface. Upon internalization of an antigen, an APC can display antigen-class I and II major histocompatibility complex (MHC) on the membrane together with co-stimulatory signals to activate antigen-specific T cells (*e.g.*, tumor antigen-specific T cells), which can lead to an adaptive immune response.

[0074] As used herein, the terms “Cas protein,” “Cas nuclease” and “Cas molecule” are interchangeable and refer to the enzyme responsible for cutting DNA in the CRISPR/Cas system. It can include enzymes from type I, II, and III CRISPR/Cas systems (*e.g.*, Cas3, Cas9, Cas10, Cas12, etc). “Cas9 protein” generally refers to the enzyme from the bacterial type II CRISPR/Cas system responsible for cutting DNA. Cas9 can include wild-type proteins and functional mutants thereof. “Cas12 protein” generally refers to the enzyme from the bacterial type II CRISPR/Cas system responsible for cutting DNA. Cas12 can include wild-type proteins and functional mutants thereof. For instance, in some instances, the Cas12 is a Cas12a protein, also known as Cpf1.

[0075] As used herein, the terms “chimeric antigen receptor” and “CAR” are used interchangeably to refer to an engineered receptor that grafts an arbitrary specificity onto an immune effector cell, such as a T cell (*e.g.*, cytotoxic T cell). A CAR is typically a fusion protein comprising antigen recognition moieties and cell-activation elements. For instance, a CAR may have an extracellular domain (ectodomain), which comprises an antigen-binding domain and a stalk region, a transmembrane domain and an intracellular (endodomain) domain. A chimeric antigen receptor is also known as an artificial T cell receptor, a chimeric T cell receptor, or a chimeric immunoreceptor.

[0076] As used herein, the term “CRISPR/Cas system” refers to a group of molecules including RNA-guided nucleases or other effector molecules and guide RNA (gRNA) molecules, which can direct and implement RNA-guided nucleases or other effector molecules to a target nucleic acid. The target nucleic acid is modified by the interaction of the CRISPR/Cas system and a sequence present in the target nucleic acid, for example, to cause cleavage (*e.g.*, hydrolysis of one or more phosphodiester bonds) of the target nucleic acid. The CRISPR/Cas system can be used for introducing genetic alterations into a cell (*e.g.*, adding, disrupting or changing the sequence of specific genes) as well as altering gene regulation of genes. For instance, the CRISPR/Cas system can be used to integrate nucleic acid sequences encoding a gene of interest into specific regions of the genome. In some instances, the CRISPR system comprises gRNA and Cas protein, for example, a Cas9 or a Cas12 protein. A system containing Cas9 or a functional mutant thereof is referred to as the “Cas9 system” or “CRISPR/Cas9 system” in this application. A system containing Cas12 or a functional mutant thereof is referred to as the “Cas12 system” or “CRISPR/Cas12 system” in

this application. In some instances, gRNA molecules and Cas molecules can be complexed to form a ribonucleoprotein (RNP) complex.

[0077] As used herein, the term “co-stimulatory ligand” or “co-stimulatory molecule” refers to a molecule that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a co-stimulatory signal which, in addition to the primary signal provided by, for instance, binding of a T cell receptor (TCR) an MHC molecule loaded with an antigen, leads to an T cell-mediated response. A co-stimulatory ligand can include, but is not limited to, CD2, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD58, CD70, CD83, CD86, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor (LTBR), 3TR6, ILT3, ILT4, HVEM, BTLA, an agonist or antibody that binds a Toll ligand receptor and a ligand that specifically binds with B7-H3.

[0078] As used herein, the term “dosage form” refers to a discrete amount of a composition comprising a predetermined amount of the active ingredient (*e.g.*, a population of cells). The amount of the active ingredient is generally equal to the dosage of the active ingredient that would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage. The relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and any additional ingredients in a pharmaceutical composition will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. The dosage form can further include one or more additional pharmaceutically active agents. In some cases, the dosage form is for administration by injection into a subject.

[0079] As used herein, the term “differentiation,” and its grammatical equivalents, refers to a process by which a stem cell or progenitor cell alters from one cell type to a more specialized cell type. Each specialized cell type in an organism can express a subset of all the genes that constitute the genome of the cell. Each cell type can be defined by its particular pattern of regulated gene expression. Cell differentiation can thus be described as a transition of a cell from one cell type to another cell type coincident with a switch from one pattern of gene expression to another.

[0080] As used herein, the term “encoding” refers to the property of specific sequences of nucleotides in a nucleic acid, such as a gene, a cDNA, or an mRNA, to serve as templates for

synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene or at least the exons of a gene encodes a protein (*e.g.*, IL-12) if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

[0081] As used herein, the term “expression” refers to the transcription and/or translation of a particular nucleotide sequence in a cell. Similarly, as used herein, the term “overexpress” refers to a level of expression of a gene in a cell under particular conditions that is increased relative to the level of expression in a cell under normal conditions.

[0082] As used herein, the terms “enhance” and “increase” are interchangeable and refer to any statistically significant increase in biological activity (*e.g.*, an immune response). For example, an enhancement or increase in biological activity can refer to an increase of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% in biological activity.

[0083] As used herein, the term “genetic modification” or “genetic alteration” refers to a change at the DNA level of a cell. A genetic modification includes an insertion, deletion, or substitution, typically within a defined sequence or genomic locus. In some instances, the genetic modification includes the integration of a nucleotide sequence heterologous to the genomic locus. The genetic modification can be at a single nucleotide position or at multiple nucleotides, *e.g.*, 2, 3, 4, 5 or more nucleotides, typically in close proximity to each other, *e.g.*, contiguous nucleotides. Depending on the nature of the genetic modification, the expression of the gene can be up-regulated or down-regulated.

[0084] As used herein, the term “genetically modified” or “engineered” cell refers to a cell (*e.g.*, an immune cell) that includes one or more genetic modifications, and which is not found in nature. The engineered cells can be made by any method known in the art, such as by manipulating the genome of the cell or inserting a new nucleic acid into the cell. For instance, a cell can be modified by integrating a nucleic acid encoding a gene of interest into a cell using a genome editing technique, such as a CRISPR/Cas9 system.

[0085] As used herein, the terms “gRNA molecule,” “guide RNA,” and “gRNA” refer to an RNA that functions as a guide for an endonuclease (*e.g.*, a Cas enzyme), with which it forms a complex. In certain instances, the hybridization of a part of the gRNA with DNA (for example, through the gRNA steering domain) and the binding of a part of the gRNA molecule to RNA-guided nucleases or other effector molecules (for example, at least through tracrRNA). In some instances, the gRNA molecule is composed of a single continuous polynucleotide molecule, referred to herein as a “single guide RNA” or “sgRNA” or the like.

[0086] As used herein, the term “heterologous,” when used in reference to a nucleic acid and/or polypeptide that is introduced to a host cell, refers a nucleic acid and/or polypeptide that is not naturally found in the host cell or naturally found at a given position in the genome of the host cells. For example, a construct is heterologous to a host cell if it contains some homologous sequences arranged in a manner not found in the host cell and/or the construct contains some heterologous sequences not found in the host cell.

[0087] As used herein, the term “IL-12” refers to a protein encoded by an interleukin 12 gene. IL-12 is a heterodimeric cytokine with multiple biological effects on the immune system, such as maturation of antigen presenting cells, increased presentation of antigen, and lymphocyte activation. It is composed of two subunits, p35 and p40, both of which are required for the secretion of the active form of IL-12, p70. In some cases, the IL-12 can be an engineered IL-12, such as for example, a variant IL-12 or an IL-12 that includes a fusion between the p35 and p40 subunits. The following IL-12 amino acid sequence has been introduced into the engineered cells:

MCHQQLVISWFSVLFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEED
GITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLHKKKEDGIWS
TDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTC
GAATLSAERVVRGDNKEYEYSVEQEDSACPAAEESLPIEVMVDAVHKLKYENYTSS
FFIRDIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKRE
KKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSVPGVGPVGAR
NLPVATPDPGMFPCLLHHSQNLLRAVSNNMLQKARQTLEFYPTCTSEEIDHEDITKDKTS
TVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEF
KTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKL
CILLHAFRIRAVTIDRVMSYLNAS* (SEQ ID NO: 1).

[0088] As used herein, the term “immune cell” refers to a cell of hematopoietic origin functionally involved in the initiation and/or execution of an immune response in an organism. An immune cell can be part of the innate and/or adaptive immune system. Exemplary immune cells include, but are not limited to, cells of the myeloid lineage (*e.g.*, neutrophils, dendritic cells, eosinophils, mast cells, basophils, monocytes, microglia, and precursors thereof), as well as cells of the lymphoid lineage (*e.g.*, T cells, B cells, natural killer cells, and precursors thereof).

[0089] As used herein, the term “immune response” refers to the physiological reaction that occurs in an organism in response to exogenous factors, pathogens, injury, or a disease (*e.g.*, cancer). An immune response can include activation of either or both the adaptive and innate immune system. An “adaptive immune response” refers to an immune response specific to a particular antigen (*e.g.*, a tumor antigen) that involves the formation of antibodies and/or the activation of lymphocytes to remove the antigen.

[0090] As used herein, the term “induced pluripotent stem cell” or “iPSC” refers to any pluripotent stem cell artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a “forced” expression of specific genes. A “pluripotent” cell is a cell that has the ability to differentiate into cells from any of the three germ layers of an organism (endoderm, mesoderm or ectoderm), but not into extra-embryonic tissues like the placenta. The pluripotency may be incomplete or partial, in that the pluripotent cell may form cells of all three germ layers but may not exhibit all the characteristics of completely pluripotent cells.

[0091] As used herein, the term “instructional material” refers to a publication, a recording, a diagram, or any other medium of expression that can be used to communicate the usefulness of the compositions and methods of using the compositions associated with the publication, recording, diagram or other medium of expression. The instructional material of a kit of the disclosure can, for example, be affixed to a container that contains the population of cells and/or pharmaceutical composition of the disclosure, or be shipped together with a container that contains the population of cells and/or pharmaceutical composition. Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the compositions be used cooperatively by the recipient.

[0092] As used herein, the terms “MHC”, “MHC protein,” and “MHC molecule” are used interchangeably to refer to a major histocompatibility complex molecule. An MHC binds to and presents antigens to immune effector cells, such as T cells (*e.g.*, cytotoxic T cells), by interacting with a T cell receptor, and are essential for an adaptive immune response. In some cases, the MHC molecule may be a MHC class I molecule or a MHC class II molecule. MHC class I molecules include an α -chain and a β_2m chain, and are encoded by the HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-G genes in humans. MHC class II molecules include an α -chain and a β chain, and are encoded by the HLA-DP, HLA-DQ, and HLA-DR genes in humans. An MHC molecule of the disclose may be an engineered MHC molecule. For example, an engineered MHC molecule comprised of a single chain fusion of the MHC chains. An engineered MHC molecule may also include an antigen-presenting polypeptide covalently or non-covalently bound to the MHC molecule.

[0093] As used herein, the term “myeloid progenitor cell” refers to a cell capable of dividing and/or undergoing differentiation into one or more mature myeloid cells. A myeloid progenitor may differentiate into one or more myeloid cell types, including, but not limited to, monocytes, microglia, macrophages, dendritic cells, basophils, eosinophils, erythrocytes, mast cells, neutrophils, megakaryocytes, or platelets, or any intermediate progenitor thereof.

[0094] As used herein, the term “nucleic acid” or “nucleic acid molecule” refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (*e.g.*, enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages.

Analogues of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like.

[0095] As used herein, the terms “patient,” “subject,” “individual,” and the like are used interchangeably and refer to any animal, or cells thereof, whether in vitro or in situ, amenable to the compositions and methods described herein. In some instances, the patient, subject or individual is a human.

[0096] As used herein, the term “pharmaceutically acceptable excipient, carrier or diluent” refers to any material which, when combined with an active ingredient (*e.g.*, a population of cells), allows the ingredient to retain biological activity and is non-reactive with the subject’s immune system. Examples include, but are not limited to, any of the standard pharmaceutical excipients, carriers, or diluents, such as a phosphate buffered saline solution, normal saline, water, emulsions such as oil/water emulsion, and various types of wetting agents.

[0097] As used herein, the term “progenitor cell,” and its grammatical equivalents, refers to a descendant of a stem cell that can further differentiate into specialized cell types within a particular cell lineage. For example, a progenitor cell can be a lymphoid progenitor cell, whereby upon further differentiation give rise to a lymphoblast (the precursor to T cells), or a myeloid progenitor cell, whereby upon further differentiation give rise to a myeloblast (*e.g.*, the precursor to granulocytes).

[0098] As used herein, the term “promoter”, and its grammatical equivalents, refers to a region of a nucleic acid positioned upstream of a gene where relevant proteins (such as RNA polymerase and transcription factors) bind to initiate transcription of the gene. The promoter can be the promoter of an endogenous house-keeping gene (*e.g.*, GAPDH). The promoter can be a tissue-specific promoter. For example, a promoter of a gene that is turned on or off in certain cell or tissue types.

[0099] As used herein, the term “substantially” or “essentially” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or higher compared to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In some instances, the terms “essentially the same” or “substantially the

same” refer a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that is about the same as a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

[00100] As used herein, the term “sustained transcriptionally active payload region” or “STAPLR” and its grammatical equivalents, refers to an intergenic region (*i.e.*, a stretch of nucleotide sequences located between two neighboring genes) in the mammalian genome that allows consistent levels of expression of nucleic acids encoding a protein of interest (*e.g.*, IL-12) integrated therein, including as the cell undergoes changes in its differentiation state. STAPLR expands the repertoire of genomic safe harbors where nucleic acids encoding a protein of interest (*e.g.*, IL-12) can be stably integrated and their expression can be maintained over multiple passages and as the cell changes its phenotype. The term “payload” or “genomic payload” in the context of STAPLR refers to exogenous or heterologous nucleotide sequences introduced to the region. The STAPLR can refer to an intergenic region found between essential genes or genes that are expressed throughout different cell states.

[00101] As used herein, the term “sustained transgene expression locus” or “STEL” refer to a locus in the genome of an organism that is resistant to silencing of gene expression. For instance, a STEL can be resistant to silencing over time or after changes in cell fate (*e.g.*, differentiation), such that expression of genes contained in the STEL is sustained. Exemplary STEL include, but are not limited to genes encoding ribosomal subunits, mitochondria proteins, actin proteins, eukaryotic translation factors, and histones. Additional exemplary STEL are described in WO2021072329A1, which is incorporated herein by reference.

[00102] As used herein, the term “targeting moiety” refers to a nucleic acid, polypeptide, peptide, glycoprotein, glycopeptide, proteoglycan, carbohydrate, lipid, small molecule, etc., which is present on the surface of a cell and permits binding of the cell to one or more “receptors,” “targets,” or “markers” associated with a particular organ, tissue, or cell in an organism. Exemplary targeting moieties include, but are not limited to, chimeric antigen receptors (CARs), T-cell receptors (TCRs), and receptors for a cell surface molecule operably linked through at least a transmembrane domain in an internal signaling domain capable of activating a T cell upon binding of the extracellular receptor portion of a protein.

[00103] As used herein, the term “T cell” or “T lymphocyte” refers to a type of lymphocyte that plays a central role in cell-mediated immunity. T cells may be distinguished

from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface. Exemplary T cell types include, but are not limited to, conventional adaptive T cells, which include helper CD4⁺ T cells (also known as helper T cells), CD8⁺ T cells (also known as cytotoxic T cells), memory T cells, and regulatory CD4⁺ T cells (also known as T regs), and innate-like T cells including natural killer T cells (also known as NKT cells), mucosal associated invariant T cells, and gamma delta T cells. T cells can be naturally occurring or non-natural, *e.g.*, modified T cells, such as CAR-T cells.

[00104] As used herein, the term “T cell activation” refers to activation of CD8⁺ T cells, activation of CD4⁺ T cells, stimulation of cytotoxic activity of T cells, stimulation of cytokine secretion by T cells, detectable effector functions, modification of the differentiation state of a T cell (*e.g.*, promote expansion and differentiation from T effector to T memory cell), and/or any combination thereof. T cell activation is mediated through stimulation of the TCR receptor by antigen-loaded MHC molecules. The term “activated T cells” refers to, among other things, T cells that are undergoing cell division.

[00105] As used herein, the term “T cell-mediated immune response” refers to an immune response associated with the activation of T cells (*e.g.*, CD8⁺ T cells) in response to an antigen (*e.g.*, a tumor antigen).

[00106] As used herein, the term “cytotoxic T cell” or “CD8⁺ T cell” refers to a T cell that expresses the CD8 glycoprotein in the cell surface and function to destroy virus-infected cells and tumor cells. CD8⁺ T cells recognize their targets by binding to antigen (*e.g.*, a tumor antigen) associated with MHC class I molecules, which are present on the surface of all nucleated cells.

[00107] As used herein, the term “helper T cell” or “helper CD4⁺ T cell” refers to a T cell that expresses the CD4 glycoprotein on the cell surface and assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and memory B cells, and activation of cytotoxic T cells and macrophages. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules, which are expressed on the surface of antigen-presenting cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response.

[00108] As used herein, the term “therapeutic” refers to a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

[00109] As used herein, the term “treat,” or a grammatical equivalent thereof, refers to a means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder (*e.g.*, a tumor) experienced by a subject.

[00110] As used herein, the terms “tumor” and “cancer” refer to a group of diseases characterized by the rapid and uncontrolled growth of aberrant cells. Tumor cells or cancer cells can spread locally, or can become metastatic, travelling through the bloodstream and lymphatic system to invade and spread in other parts of the body. Examples of various cancers include but are not limited to, ACUTE lymphoblastic leukemia (ALL), ACUTE myeloid leukemia (AML), anal cancer, bile duct cancer, bladder cancer, bone cancer, bowel cancer, brain tumor (*e.g.*, glioblastoma), breast cancer, cancer of unknown primary, cancer spread to bone, cancer spread to brain, cancer spread to liver, cancer spread to lung, carcinoid, cervical cancer, choriocarcinoma, chronic lymphocytic leukemia (CLL), chronic myeloid leukemia (CML), colon cancer, colorectal cancer, endometrial cancer, eye cancer, gallbladder cancer, gastric cancer, gestational trophoblastic tumor (GTT), hairy cell leukemia, head and neck cancer, Hodgkin lymphoma, kidney cancer, laryngeal cancer, leukemia, liver cancer, lung cancer, lymphoma, melanoma skin cancer, mesothelioma, men’s cancer, molar pregnancy, mouth and oropharyngeal cancer, myeloma, nasal and sinus cancers, nasopharyngeal cancer, non-Hodgkin lymphoma (NHL), esophageal cancer, ovarian cancer, pancreatic cancer, penile cancer, prostate cancer, rare cancers, rectal cancer, salivary gland cancer, secondary cancers, skin cancer (non-melanoma), soft tissue sarcoma, stomach cancer, testicular cancer, thyroid cancer, unknown primary cancer, uterine cancer, vaginal cancer, and vulval cancer.

[00111] As used herein, the term “tumor antigen” refers to any antigenic substance produced or overexpressed in, by, or on the surface of tumor cells. In some instances, a tumor antigen can trigger an immune response to the tumor in the host. In other cases, the tumor antigens can be proteins that are expressed by both healthy and tumor cells, but identify a particular tumor type.

[00112] As used herein, a statement that a cell or population of cells is “positive” for a particular marker, or “expresses” a particular marker, refers to the detectable presence on or

in the cell of a particular marker, for example, a surface marker or an intracellular marker, such as transcription factors. When referring to a surface marker, the term refers to the presence of surface expression as detected by flow cytometry, for example, by staining with an antibody that specifically binds to the marker and detecting said antibody, wherein the staining is detectable by flow cytometry at a level substantially above the staining detected carrying out the same procedure with an isotype-matched control or fluorescence minus one (FMO) gating control under otherwise identical conditions and/or at a level substantially similar to that for cell known to be positive for the marker, and/or at a level substantially higher than that for a cell known to be negative for the marker.

[00113] As used herein, a statement that a cell or population of cells is “negative” for a particular marker, or fails to express a particular marker or gene, refers to the absence of substantial detectable presence on or in the cell of a particular marker for example, a surface marker or an intracellular marker, such as transcription factors. When referring to a surface marker, the term refers to the absence of surface expression as detected by flow cytometry, for example, by staining with an antibody that specifically binds to the marker and detecting said antibody, wherein the staining is not detected by flow cytometry at a level substantially above the staining detected carrying out the same procedure with an isotype-matched control or fluorescence minus one (FMO) gating control under otherwise identical conditions, and/or at a level substantially lower than that for cell known to be positive for the marker, or at a level substantially similar as compared to that for a cell known to be negative for the marker.

[00114] Throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

II. Population of engineered cells

[00115] In some aspects of the disclosure, provided herein are a population of engineered cells that secrete a cytokine. In some embodiments, the cytokine is $\text{TNF}\alpha$, IL-1RA, IL-1 β , IL-

1 α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-20, IL-21, IL-23, IL-33, IL-37, TRAIL, MIF, TGF β , IFN α , IFN γ , or GM-CSF. In some embodiments, the cytokine is interleukin-12 (IL-12).

[00116] In some embodiments, the population of engineered cells that secrete a cytokine is a population of engineered immune cells that secrete a cytokine. In some embodiments, the population of engineered immune cells that secrete the cytokine is a population of engineered immune cells that secrete IL-12. In some embodiments, the population of engineered immune cells includes one or more of neutrophils, dendritic cells, eosinophils, mast cells, basophils, monocytes, microglia, T cells, B cells, natural killer cells, or any precursors or progenitors thereof. In some embodiments, the population of engineered immune cells includes one or more of myeloid progenitor cells, microglia precursor cells, microglial cells, T cells, natural killer cells, or macrophages. In some embodiments, the population of engineered cells comprises a population of pluripotent stem cells. For example, the population of engineered cells may comprise a population of induced pluripotent stem cells.

[00117] In some embodiments, the population of engineered immune cells secretes the cytokine at a concentration between 1.0 and 1,000 ng/ml, as measured by homogeneous time resolved fluorescence (HTRF), when cultured (*in vitro*) at a density of 1 million cells per milliliter for 24 hours. Exemplary concentration ranges within this spectrum include, but are not limited to: between 1.0 - 10.0 ng/mL, between 10 - 50 ng/mL, between 50 - 100 ng/mL, between 100 - 200 ng/mL, between 200 - 300 ng/mL, between 300 - 400 ng/mL, between 400 - 500 ng/mL, between 500 - 600 ng/mL, between 600 - 700 ng/mL, between 700 - 800 ng/mL, between 800 - 900 ng/mL, and between 900 - 1,000 ng/mL. In some embodiments, the populations of engineered immune cells secrete the cytokine at a concentration of less than 1.0 ng/ml, when cultured (*in vitro*) at a density of 1 million cells per milliliter for 24 hours. For example, in some embodiments, the population of engineered immune cells secrete IL-12 at a concentration of between 0.01 - 1.0 ng/mL, between 0.5 - 1.0 ng/mL, or between 0.01 - 0.5 ng/mL. In some embodiments, the population of engineered immune cells secrete the cytokine at a concentration of greater than 1,000 ng/ml when cultured (*in vitro*) at a density of 1 million cells per milliliter for 24 hours. For example, in some embodiments, the population of engineered immune cells secrete the cytokine at a concentration of between 1,000 ng/ml and 2,000 ng/ml. For example, in some embodiments, the population of engineered immune cells secrete IL-12 at a concentration of between 1,000 ng/ml and 1,500

ng/ml, or between 1,500 ng/ml and 2,000 ng/ml. In some embodiments, the cytokine is TNF α , IL-1RA, IL-1 β , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-20, IL-21, IL-23, IL-33, IL-37, TRAIL, MIF, TGF β , IFN α , IFN γ , or GM-CSF. In some embodiments, the cytokine is IL-12. Exemplary HTRF assays that can be used to assay the secretion of cytokines, such as IL-12, and other molecules described here are exemplified in the Examples and well known in the art as described in, for example, Degorce, F., *et al.* (2009) *Chem Genomics* 3:22-32; and Achard, S, *et al.* (2003) *Assay and Drug Development Technologies 1.1*, Supplement 2: 181-185, which are incorporated herein by reference.

[00118] In some embodiments, the population of engineered immune cells secretes the cytokine at a concentration that is below 1000 ng/ml, as measured by HTRF, when cultured at a density of 1 million cells per milliliter for 24 hours. For example, in some embodiments the population of engineered immune cells secretes the cytokine at a concentration that is below 900 ng/ml, below 800 ng/ml, below 700 ng/ml, below 600 ng/ml, below 500 ng/ml, below 400 ng/ml, below 300 ng/ml, below 200 ng/ml, below 100 ng/ml, below 50 ng/ml, below 10 ng/ml, or below 1 ng/ml, when cultured at a density of 1 million cells per milliliter for 24 hours. In some embodiments, the population of engineered immune cells secretes the cytokine at a concentration that is below 500 ng/ml, as measured by HTRF, when cultured at a density of 1 million cells per milliliter for 24 hours. In some embodiments, the cytokine is IL-12

[00119] In some embodiments, the population of engineered immune cells secretes IL-12 at a concentration between 1.0 and 1,000 ng/ml, as measured by HTRF, when cultured (*in vitro*) at a density of 1 million cells per milliliter for 24 hours. Examples of possible concentration ranges within this spectrum include, but are not limited to: between 1.0 - 10.0 ng/mL, between 10 - 50 ng/mL, between 50 - 100 ng/mL, between 100 - 200 ng/mL, between 200 - 300 ng/mL, between 300 - 400 ng/mL, between 400 - 500 ng/mL, between 500 - 600 ng/mL, between 600 - 700 ng/mL, between 700 - 800 ng/mL, between 800 - 900 ng/mL, and between 900 - 1,000 ng/mL. In some embodiments, the populations of engineered immune cells secrete IL-12 at a concentration of less than 1.0 ng/ml, when cultured (*in vitro*) at a density of 1 million cells per milliliter for 24 hours. For example, in some embodiments, the population of engineered immune cells secrete IL-12 at a concentration of between 0.01 - 1.0 ng/mL, between 0.5 - 1.0 ng/mL, or between 0.01 - 0.5

ng/mL. In some embodiments, the population of engineered immune cells secrete IL-12 at a concentration of greater than 1,000 ng/ml when cultured (in vitro) at a density of 1 million cells per milliliter for 24 hours. For example, in some embodiments, the population of engineered immune cells secrete IL-12 at a concentration of between 1,000 ng/ml and 2,000 ng/ml. For example, in some embodiments, the population of engineered immune cells secrete IL-12 at a concentration of between 1,000 ng/ml and 1,500 ng/ml, or between 1,500 ng/ml and 2,000 ng/ml.

[00120] In some embodiments, the population of engineered immune cells secretes IL-12 at a concentration that is below 1,000 ng/ml, as measured by HTRF, when cultured at a density of 1 million cells per milliliter for 24 hours. For example, in some embodiments the population of engineered immune cells secretes IL-12 at a concentration that is below 900 ng/ml, below 800 ng/ml, below 700 ng/ml, below 600 ng/ml, below 500 ng/ml, below 400 ng/ml, below 300 ng/ml, below 200 ng/ml, below 100 ng/ml, below 50 ng/ml, below 10 ng/ml, or below 1 ng/ml, when cultured at a density of 1 million cells per milliliter for 24 hours.

[00121] In other embodiments, the population of engineered cells that secrete the cytokine is a population of engineered myeloid progenitor cells that secrete the cytokine. In some embodiments, the population of engineered myeloid progenitor cells has a genetic modification to elicit or enhance an amount of the cytokine (*e.g.*, IL-12) that is secreted by the engineered myeloid progenitor cells as compared to myeloid progenitor cells not comprising the genetic modification. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 2 and 1000 ng/mL as measured by HTRF, when the cells are cultured at a density of 1 million cells per 24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete cytokine at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at below 1ng/mL/1M cells/24hr.

[00122] In some embodiments, the population of engineered myeloid progenitor cells that secrete the cytokine is a population of engineered myeloid progenitor cells that secrete IL-12. In some embodiments, the population of engineered myeloid progenitor cells has a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the engineered myeloid progenitor cells as compared to myeloid progenitor cells not comprising the genetic modification. In some embodiments, the population of engineered myeloid progenitor cells

secrete IL-12 at between 2 and 1000 ng/mL as measured by HTRF, when the cells are cultured at a density of 1 million cells per 24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at below 1ng/mL/1M cells/24hr.

[00123] In some embodiments, the population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) secretes IL-12 that has an amino acid sequence as set forth in SEQ ID NO: 1. In some embodiments, the IL-12 secreted by the population of engineered cells has an amino acid sequence that has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or is identical to the amino acid sequence set forth in SEQ ID NO: 1. In some embodiments, the IL-12 secreted by the population of engineered cells is a human IL-12. In some embodiments, the IL-12 secreted by the population of engineered cells is an engineered or variant IL-12. In some embodiments, a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) has a genetic modification to elicit or enhance an amount of the cytokine (*e.g.*, IL-12) that is secreted by the cells. In some embodiments, the genetic modification enhances the expression, stability or secretion of the cytokine. In some embodiments, the genetic modification to elicit or enhance an amount of the cytokine that is secreted by the cells includes an integration of a heterologous nucleic acid encoding the cytokine into the genome of the cells. In some embodiments, the integration of a heterologous nucleic acid encoding the cytokine into the genome of the cells results in stable expression and/or secretion of cytokine by the cells after proliferation or differentiation of the cells. In some embodiments, the cytokine is IL-12.

[00124] In some embodiments, a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) has a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the cells. In some embodiments, a population of engineered cells described (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) has a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the cells as compared to cells not having the genetic modification. In some embodiments, the population of engineered cells having the genetic modification secrete an amount of IL-12 that is at least

10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, or higher as compared to the amount of IL-12 secreted by cells not having the genetic modification. In some embodiments, the population of engineered cells having the genetic modification secrete an amount of IL-12 that is at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, or higher as compared to the among of IL-12 secreted by cells not having the genetic modification. In some embodiments, the genetic modification enhances the expression, stability or secretion of the IL-12.

In some embodiments, the genetic modification to elicit or enhance an amount of IL-12 that is secreted by the cells includes an integration of a heterologous nucleic acid encoding IL-12 into the genome of the cells. In some embodiments, the integration of a heterologous nucleic acid encoding IL-12 into the genome of the cells results in stable expression and/or secretion of IL-12 by the cells after proliferation or differentiation of the cells.

[00125] In some embodiments, the genetic modification to elicit or enhance an amount of IL-12 that is secreted by the cells includes an integration of a heterologous nucleic acid encoding the cytokine (*e.g.*, IL-12) into a sustained transgene expression locus (STEL) in the genome of the cells. In some embodiments, the STEL is a gene locus that encodes a protein involved in one or more of: ribonucleoprotein complex formation, focal adhesion, cell-substrate adherens junction, cell-substrate junction, cell anchoring, extracellular exosome, extracellular vesicle, intracellular organelle, anchoring junction, RNA binding, nucleic acid binding (*e.g.*, rRNA or mRNA binding), and protein binding. In some embodiments, the STEL is GAPDH. In some embodiments, the STEL is a ribosomal protein gene locus, such as an RPL or RPS gene locus. Examples of RPL genes are RPL10, RPL13, RPS18, RPL3, RPLP1, RPL13A, RPL15, RPL41, RPL11, RPL32, RPL18A, RPL19, RPL28, RPL29, RPL9, RPL8, RPL6, RPL 18, RPL7, RPL7A, RPL21, RPL37A, RPL12, RPL5, RPL34, RPL35A, RPL30, RPL24, RPL39, RPL37, RPL14, RPL27A, RPLP2, RPLP0, RPL23A, RPL26, RPL36, RPL35, RPL23, RPL4, and RPL22. Examples of RPS genes are RPS2, RPS 19, RPS 14, RPS3A, RPS 12, RPS3, RPS6, RPS23, RPS27A, RPS8, RPS4X, RPS7, RPS24, RPS27, RPS15A, RPS9, RPS28, RPS 13, RPSA, RPS5, RPS16, RPS25, RPS15, RPS20, and RPSII. In some embodiments, the STEL is a gene locus encoding a mitochondrial protein, such as MT-COI, MT-C02, MT-ND4, MT-ND1, and MT-ND2. In some embodiments, the STEL is a gene locus encoding an actin protein, such as ACTG1 and ACTB. In some embodiments, the

STEL is a gene locus encoding a eukaryotic translation elongation factor, such as EEF1A1 and EEF2, or a eukaryotic translation initiation factor such as EIEI. In some embodiments, the STEL is a gene locus encoding a histone, such as H3F3A and H3F3B. In some embodiments, the STEL is a gene locus selected from FTL, FTH1, TPT1, IMSB10, GAPDH, PTMA, GNB2L1, NACA, YBX1, NPM1, FAU, UBA52, HSP90AB1, MYL6, SERF2, and SRP14.

[00126] Advantageously, by integrating the heterologous nucleic acid encoding the cytokine (*e.g.*, IL-12) into a STEL in the genome of the cells, the payload candidate's expression cassette can be governed by an endogenous gene promoter, such as, GAPDH. Consequently, the expression of the cytokine can be linked to an endogenous gene's expression. The continued activity of the endogenous gene in the engineered cells will then imply that the linked the cytokine transgene expression will remain sustained and constitutive. In some embodiments, this disclosure provides the capability to modulate the cytokine's expression by utilizing an endogenous gene's expression as the promoter to drive transgene expression. This innovative approach enables precise control over the cytokine's secretion, allowing for fine-tuning to achieve optimal levels. By leveraging the inherent regulatory mechanisms of the endogenous gene, the disclosure offers a useful mechanism to maintain the cytokine's secretion at an appropriate and therapeutic level, thereby ensuring effective immune modulation without the risk of excessive or potentially toxic secretion.

[00127] In some embodiments, the genetic modification includes an integration of a heterologous nucleic acid encoding the cytokine (*e.g.*, IL-12) into a sustained transcriptionally active payload region (STAPLR) in the genome of the engineered cells. The STAPLRs can include open chromatin landscape for landing genomic payloads, as well as regulatory regions that enhance expression of the payload, such as promoters or enhancers. The STAPLR can be of any size, such as for example, at least 100 base pairs to at least 100,000 base pairs in length. In some embodiments, the STAPLR is in the vicinity of transcriptionally active gene. In some embodiments, the STAPLR is near a gene that is specifically expressed in the population of engineered cells. In some embodiments, the STAPLR includes the intergenic region between the RPL34 gene and the OSTC gene, the intergenic region between the ACTB gene and the FSCN1 gene, the intergenic region between the AKIRIN1 gene and the NDUFS5 gene, the intergenic region between the PRDX1 gene and the AKR1A1 gene, the intergenic region between the PTGES3 gene and

the NACA gene, the intergenic region between the MLF2 gene and the PTMS gene, the intergenic region between the RAB13 gene and the RPS27 gene, the intergenic region between the JTB gene and the RAB13 gene, the intergenic region between the AKR1A1 gene and the NASP gene, the intergenic region between the NDUFS5 gene and the MACF1 gene, the intergenic region between the SRSF9 gene and the DYNLL1 gene, the intergenic region between the MYL6B gene and the MYL6 gene, the intergenic region between the GPX1 gene and the RHOA gene, the intergenic region between the HNRNPA2B1 gene and the CBX3 gene, the intergenic region between the ROMO gene and the RBM39 gene, or the intergenic region between the PA2G4 gene and the RPL41 gene. In some embodiments, the heterologous nucleic acid encoding IL-12 integrated at a location that is at least 100-5000 base pairs away from the nearest flanking gene of the STAPLR. In some embodiments, the exogenous nucleotide sequence has been integrated at a location that is at least 100, at least 200, at least 300, at least 400, at least 500, at least 1000, at least 1500, at least 2000, at least 2500, at least 3000, at least 3500, at least 5000, at least 10000, at least 15000, or at least 20000 base pairs away from the nearest flanking gene of the STAPLR. In some embodiments, the STAPLR is located near a STEL. For additional information relating to STEL, see International Application No PCT/US2023/66396, which is incorporated by reference. For additional information relating to STAPLR, *see* International Application No. PCT/US2023/066396, which is incorporated herein by reference.

[00128] In some embodiments, the genetic modification includes an integration of a heterologous nucleic acid encoding the cytokine (*e.g.*, IL-12) into a STAPLR in the genome of the engineered cells. In some embodiments, a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) can express one or more additional cytokines. The cytokine can be any of a chemokine, interferon, interleukin, lymphokine, and tumor necrosis factor. Exemplary additional cytokines or immunomodulatory soluble factors that can be expressed by the population of engineered cells include, but are not limited to, LILRA3, sCD40L, CCLI, CCL2, CCL3, CCR4, CCL5, CCL7, CCL8/MCP-2, CCL11, CCL13 (also known as MCP-4), HCC-I/CCLI 4, CTAC/CCLI 7, CCLI9, CCL22, CCL23, CCL24, CCL26, CCL27, VEGF, PDGF, lymphotactin (also known as XCLI), Eotaxin, FGF, EGF, IP-10, TRAIL, FASL, GCP-2 (also known as CXCL6), NAP-2 (also known as CXCL7), CXCL8, CXCL10, ITAC (also known as CXCL11), CXCL12, CXCL13, CXCL15, TGFBR11, TGFb, IL-1a, IL1b,

IL1Rn, IL-6, IL-7, IL-15, IL-16, IL-2, IL-10, IL-18, IL-18 binding protein, IL-21, IFN α , IFN β , IFN γ , TNF α , or any other cytokine known in the art.

[00129] In some embodiments, a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) is able to present antigens to T cells. In other embodiments, the T cells are CD4⁺ T cells. In some embodiments, the T cells are modified T cells. In some embodiments, the modified T cells are CAR-T cells. In some embodiments, the presentation of tumor antigens by the population of engineered cells results in activation of the T cells. In some embodiments, the presentation of tumor antigens by the population of engineered cells results in production of tumor antigen-specific T cells.

[00130] In some embodiments, a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) can activate or enhance an immune response when administered to a subject. For instance, a population of engineered cells can activate or enhance an immune response to an antigen (*e.g.*, a tumor antigen) when administered to a subject. In some embodiments, the population of cells results in the activation or enhancement of an immune response to an antigen (*e.g.*, a tumor antigen) when administered to a subject. In some embodiments, the population of engineered cells results in the activation or enhancement of a T-cell mediated immune response to an antigen when administered to a subject. In some embodiments, the T cell-mediated immune response is a CD8⁺ T cell-mediated immune response. In other embodiments, the T cell-mediated immune response is a CD4⁺ T cell-mediated immune response. In some embodiments, the subject has a tumor, and the antigen is a tumor antigen.

[00131] In some embodiments, the population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) can further express cell surface proteins that enhance presentation of antigen (*e.g.*, tumor antigen) to T cells and/or the immune triggered by interacting with the T cells. For instance, the population of engineered cells can express cell surface proteins associated with antigen presenting cells (APCs) that facilitate presentation of antigens (*e.g.*, tumor antigens) to T cells and B cells, thereby enhancing an immune response to said antigens.

[00132] In some embodiments, each of the engineered cells in the populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered

myeloid progenitor cells) described herein has a major histocompatibility complex (MHC) molecule on the cell surface. In some embodiments, the MHC molecule binds to and presents an antigen to T cells. In some embodiments, the MHC molecule is an MHC class I molecule. In other embodiments, the MHC molecule is an MHC class II molecule. In some embodiments, each of the engineered cells overexpress the MHC molecule on the cell surface. In some embodiments, the overexpression of the MHC molecule on the cell surface increases the presentation of the antigens to T cells.

[00133] In some embodiments, each of the engineered cells in the populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) described herein has a co-stimulatory molecule on the cell surface. The co-stimulatory molecule can be any co-stimulatory molecule that can interact with a protein in the cell surface of T cells to enhance an immune response. In some embodiments, the each of the engineered immune cells or engineered myeloid progenitor cells express a an immune modulatory molecule selected from CD2, CD7, B7-1 (CD80), B7-2 (CD86), 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD58, CD70, CD80, CD83, CD86, HLA-G, HLA-E, MICA, MICB, HVEM, lymphotoxin beta receptor, 3TR6, ILT3, ILT4, TGFb, IL-1Rn, HVEM, BTLA, an agonist or antibody that binds to a Toll ligand receptor, and B7-H3 ligand. In some embodiments, the said co-stimulatory molecule is CD80, CD86, or CD40. In some embodiments, the co-stimulatory molecule enhances the immune response triggered by the population of engineered cells. In some embodiments, the co-stimulatory molecule enhances a T cell-mediated immune response by interacting with T cells, *e.g.*, by interacting with a cell surface protein expressed by the T cells. In some embodiments, the T cell-mediated immune response is a CD8⁺ T cell mediated response. In other embodiments, the T cell-mediated immune response is a CD4⁺ T cell mediated response.

[00134] In some embodiments, each of the engineered cells in the populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) described herein has a targeting moiety on the cell surface. The targeting moiety can allow the engineered immune cells of the myeloid progenitor cells to specifically interact with a target cell, tissue or organ in a subject. For instance, the targeting moiety can promote the interaction of the engineered immune cells of the myeloid progenitor cells with T cells, thereby enhancing presentation of antigens (*e.g.*, tumor antigens) and/or the

activation of a T cell-mediated immune. In some embodiments, the targeting moiety binds to a protein expressed in the cell surface of T cells. In some embodiments, the targeting moiety binds to a protein expressed in the cell surface of CD8⁺ T cells. In other embodiments, the targeting moiety binds to a protein expressed in the cell surface of CD4⁺ T cells. In some embodiments, the targeting moiety is an engineered receptor. In some embodiments, the targeting moiety is a CAR.

[00135] In some embodiments, the population of engineered cells is a population of human engineered cells.

Methods of preparing a population of engineered cells

[00136] In some aspects of the disclosure, provided herein are methods of preparing any of the populations of engineered cells described herein.

[00137] In some embodiments, the method to prepare a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) includes introducing a genetic modification to elicit or enhance an amount of the cytokine (*e.g.*, IL-12) that is secreted by the cells into induced pluripotent stem cells (iPSCs) using a CRISPR/Cas system; and deriving the population of engineered cells from the iPSCs. In some embodiments, the genetic modification to elicit or enhance an amount of the cytokine that is secreted by the cells includes is the integration of a heterologous nucleic acid encoding the cytokine. In some embodiments, the cytokine is IL-12.

[00138] In some embodiments, introducing the genetic modification to elicit or enhance an amount of the cytokine (*e.g.*, IL-12) that is secreted by the cells into iPSCs includes integrating the heterologous nucleic acid encoding the cytokine is integrated into the genome of the iPSCs using a CRISPR/Cas system. The CRISPR/Cas system has been used for introducing genetic modifications and gene regulation in a variety of species. Without being limited by theory, a target nucleic acid can be modified by the interaction of the CRISPR/Cas system and a sequence present in the target nucleic acid, for example, to cause cleavage (*e.g.*, hydrolysis of one or more phosphodiester bonds) of the target nucleic acid and introduce the genetic modification. In some embodiments, the heterologous nucleic acid encoding IL-12 is

integrated into the genome of the iPSCs using a CRISPR/Cas9 system or a CRISPR/Cas12 system.

[00139] In some embodiments, the heterologous nucleic acid encoding the cytokine (*e.g.*, IL-1) is integrated into a STEL in the genome of the iPSCs. In some embodiments, the STEL is a gene locus that encodes a protein involved in one or more of: ribonucleoprotein complex formation, focal adhesion, cell-substrate adherens junction, cell-substrate junction, cell anchoring, extracellular exosome, extracellular vesicle, intracellular organelle, anchoring junction, RNA binding, nucleic acid binding (*e.g.*, rRNA or mRNA binding), and protein binding. In some embodiments, the STEL is GAPDH. In some embodiments, the STEL is a ribosomal protein gene locus. In some embodiments, the ribosomal protein gene locus is an RPL or RPS gene locus. In some embodiments, the RPL gene locus is RPL10, RPL13, RPS18, RPL3, RPLP1, RPL13A, RPL15, RPL41, RPL11, RPL32, RPL18A, RPL19, RPL28, RPL29, RPL9, RPL8, RPL6, RPL18, RPL7, RPL7A, RPL21, RPL37A, RPL 12, RPL5, RPL34, RPL35A, RPL30, RPL24, RPL39, RPL37, RPL14, RPL27A, RPLP2, RPLP0, RPL23A, RPL26, RPL36, RPL35, RPL23, RPL4, or RPL22. In some embodiments, the RPS gene locus is RPS2, RPS19, RPS14, RPS3A, RPS12, RPS3, RPS6, RPS23, RPS27A, RPS8, RPS4X, RPS7, RPS24, RPS27, RPS15A, RPS9, RPS28, RPS 13, RPSA, RPS5, RPS16, RPS25, RPS15, RPS20, or RPSII. In some embodiments, the STEL is a gene locus encoding a mitochondrial protein. In some embodiments, the gene locus encoding a mitochondrial protein is MT-COI, MT-C02, MT-ND4, MT-ND1, or MT-ND2. In some embodiments, the STEL is a gene locus encoding an actin protein. In some embodiments, the actin protein gene locus is ACTG1 and ACTB. In some embodiments, the STEL is a gene locus encoding a eukaryotic translation elongation factor or a eukaryotic translation initiation factor such. In some embodiments, the gene locus encoding the eukaryotic translation elongation factor or the eukaryotic translation initiation factor is EEF1A1, EEF2, or EIEI. In some embodiments, the STEL is a gene locus encoding a histone. In some embodiments, the gene locus encoding a histone is H3F3A or H3F3B. In some embodiments, the STEL is a gene locus selected from FTL, FTH1, TPT1, IMSB10, GAPDH, PTMA, GNB2L1, NACA, YBX1, NPM1, FAU, UBA52, HSP90AB1, MYL6, SERF2, and SRP14.

[00140] In some embodiments, the heterologous nucleic acid encoding the cytokine (*e.g.*, IL-12) is integrated into a STAPLR loci in the genome of the iPSCs. In some embodiments, the STAPLR is in the vicinity of transcriptionally active gene. In some embodiments, the

STAPLR is near a gene that is specifically expressed in the population of engineered cells. In some embodiments, the STAPLR includes the intergenic region between the RPL34 gene and the OSTC gene, the intergenic region between the ACTB gene and the FSCN1 gene, the intergenic region between the AKIRIN1 gene and the NDUFS5 gene, the intergenic region between the PRDX1 gene and the AKR1A1 gene, the intergenic region between the PTGES3 gene and the NACA gene, the intergenic region between the MLF2 gene and the PTMS gene, the intergenic region between the RAB13 gene and the RPS27 gene, the intergenic region between the JTB gene and the RAB13 gene, the intergenic region between the AKR1A1 gene and the NASP gene, the intergenic region between the NDUFS5 gene and the MACF1 gene, the intergenic region between the SRSF9 gene and the DYNLL1 gene, the intergenic region between the MYL6B gene and the MYL6 gene, the intergenic region between the GPX1 gene and the RHOA gene, the intergenic region between the HNRNPA2B1 gene and the CBX3 gene, the intergenic region between the ROMO gene and the RBM39 gene, or the intergenic region between the PA2G4 gene and the RPL41 gene. In some embodiments, the heterologous nucleic acid encoding IL-12 integrated at a location that is at least 100-5000 base pairs away from the nearest flanking gene of the STAPLR. In some embodiments, the STAPLR is located near a STEL.

[00141] In some embodiments, the heterologous nucleic acid encoding IL-12 further includes one or more regulatory elements. Such a regulatory element can include a regulatory sequence, which is any DNA sequence that is responsible for the regulation of gene expression, such as promoters and operators. The regulatory element can be a segment of a nucleic acid molecule, which is able to increase or decreasing the expression of specific genes within an organism.

[00142] In some embodiments, the regulatory element is a promoter. A promoter is a nucleotide sequence that directs the transcription of a structural gene. In some alternatives, a promoter is in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. Without being limiting, these promoter elements can include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee et al., Mol. Endocrinol. 7:551 (1993);), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman et al., Seminars in Cancer Biol. 1:47 (1990); incorporated by reference in its

entirety), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly et al., J. Biol. Chem. 267:19938 (1992); incorporated by reference in its entirety), AP2 (Ye et al., J. Biol. Chem. 269:25728 (1994); incorporated by reference in its entirety), SPI, cAMP response element binding protein (CREB; Loeken et al., Gene Expr. 3:253 (1993); hereby expressly incorporated by reference in its entirety) and octamer factors (see, in general, Watson et al., eds., Molecular Biology of the Gene, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987; incorporated by reference in its entirety)), and Lemaigre and Rousseau, Biochem. J. 303:1 (1994); incorporated by reference in its entirety). In some alternatives, promoters used herein can be inducible or constitutive promoters. Without being limiting, inducible promoters can include, for example, a tamoxifen inducible promoter, tetracycline inducible promoter, or a doxycycline inducible promoter (*e.g.*, tre) promoter. Constitutive promoters can include, for example, SV40, CMV, UBC, elongation factor 1 α short (EFS) promoter, EF1 α , PGK, or CAGG. Any suitable promoter known in the art for expression of a gene in a population of engineered cells as described herein can be used.

[00143] In some embodiments, the heterologous nucleic acid encoding the cytokine (*e.g.*, IL-12) is introduced into the iPSCs in a vector. The vector can be plasmid, virus, or other nucleic acid designed for introducing a nucleic acid of interest into a cell. The vector is used to introduce a gene of interest into a host cell whereby the vector will interact with polymerases in the cell to express the protein encoded in the vector. The vector can exist in the cell extra-chromosomally or integrated into the genome of the host cell. In some embodiments, a vector described herein is integrated into the genome of a cell. In some embodiments, the heterologous nucleic acid encoding the cytokine is delivered in the same vector or a separate vector as the CRISPR/Cas system.

[00144] Methods for obtaining iPSCs for use in the method for preparing a population of engineered cells as described herein are known in the art. For example, the iPSCs can be prepared by inducing expression of one or more genes, *e.g.*, POU5F1/OCT4 in combination with, but not restricted to, SOX2, KLF, c-MYC, NANOG, and/or LIN28/LIN28A. Reprogramming factors may be delivered by various means (*e.g.*, viral, non-viral, RNA, DNA, or protein delivery). Alternatively, endogenous genes may be activated by using, *e.g.*, a CRISPR/Cas system to reprogram non-pluripotent cells into iPSCs.

[00145] Methods for inducing differentiation of PSCs into cells of various lineages are well known in the art. For example, methods for inducing differentiation of PSCs into dendritic cells are described in Slukvin et al., J Imm. (2006) 176:2924-32; and Su et al., Clin Cancer Res. (2008) 14(19):6207-17; and Tseng et al., Regen Med. (2009) 4(4):513-26.

[00146] After introducing the genetic modification to elicit or enhance an amount of IL-12 that is secreted, the population of engineered cells is derived from the iPSCs. In some embodiments, deriving the population of engineered cells from the iPSCs includes differentiating the iPSCs into the engineered cells. Differentiation can be performed using suitable known methods. For example, methods for inducing PSCs into hematopoietic progenitor cells, cells of myeloid lineage, and T lymphocytes are described in, *e.g.*, Kennedy et al., Cell Rep. (2012) 2:1722-35. Exemplary methods of differentiating iPSCs into myeloid progenitor cells are described in, for instance, U.S. Patent No. 11,525,119, B2 and U.S. Patent No. 10,260,044 B1. In some embodiments, differentiating the iPSCs into the engineered cells includes contacting the iPSCs with one or more differentiation factors. The specific combination of differentiation factors used will depend on the desired cell type(s). In some embodiments, differentiating the iPSCs into the engineered cells includes contacting the iPSCs with one or more differentiation factors that drive the commitment and/or differentiation into myeloid progenitor cells. In some embodiments, the one or more differentiation factors that drive the commitment and/or differentiation into myeloid progenitor cells includes one or more of bone morphogenetic protein 4 (BMP4), stem cell factor (SCF), Fms-like tyrosine kinase 3 (FLT3/CD135), IL-6, IL-3, granulocyte colony stimulating factor (G-CSF), granulocyte and monocyte stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF).

[00147] In some embodiments, the method for preparing a population of cells described herein further include an isolation step. In some embodiments, the isolation step is for isolating cells that have the genetic modification to elicit or enhance an amount of IL-12 that is secreted, from cells lacking the genetic modification prior to deriving the population of engineered cells from the iPSCs. In other embodiments, the isolation step is for isolating derived engineered cells from iPSCs, or intermediate cells, after deriving the population of engineered cells. Any suitable method known in the art for selection and isolation of cells of interest can be used, such as centrifugation and fluorescence-activated cell sorting (FACS).

[00148] In some embodiments, the method for preparing a population of cells described herein further include expanding the population of engineered cells. In some embodiments, the population of engineered cells is expanded after isolating the engineered cells from the iPSCs. Expanding the population of engineered cells can include culturing or contacting the engineered cells with a medium having a cytokine and growth factor mixture permissive for expansion of the engineered cells.

[00149] In some embodiments, the method for preparing a population of cells described herein further includes preserving the population of engineered cells after the deriving. For instance, the population of engineered cells can be cryopreserved. The cryopreserved population of engineered cells can be thawed at a later time, and can be diluted for downstream applications.

[00150] In some embodiments, the population of engineered immune cells or the population of engineered myeloid progenitor cells produced by the methods described herein is used in preparing a composition for treating a tumor (*e.g.*, a glioblastoma) in a subject.

Differentiation of stem cells into myeloid progenitor cells

[00151] This disclosure provides a serum- and feeder-free protocol to differentiate stem cells (including human iPSCs) towards the myeloid lineage. Various methods known in the art can be employed in connection with the present disclosure. Muffat et.al, Nat Med. 2016 Nov; 22(11): 1358–1367, Pandaya et. al, Nat Neurosci. 2017 May; 20(5): 753–759, Abud et. al, Neuron 2017 Apr 19;94(2):278-293, Douvaras et. al, Stem Cell Reports, Volume 8, Issue 6, P1516-1524, June 06, 2017, Van Wilgenburg PLOS ONE, <https://doi.org/10.1371/journal.pone.0071098>--Aug 2013, Haenseler et.al, Stem Cell Reports 2017 Jun 6;8(6):1727-1742 and Takata et.al, Immunity 2017 Jul 18;47(1):183-198. The disclosures of these are incorporated herein by reference. Myeloid progenitor cells arise from the yolk sack during embryonic development. In order to mimic the embryonic development of these myeloid progenitor cells, primitive streak-like cells are generated from PSCs, followed by hematopoietic and myelopoietic cocktails in serum-free media. This results in the appearance of myeloid progenitor cells in the supernatant fraction of the culture that expressed myeloid markers, including but not limited to CD45, CD14, CX3CR1, CD33, CD11b. These myeloid cells can continue to be generated in the supernatant fraction of the culture for a considerable amount of time, often reaching 3 to 4 months. Myeloid progenitor

cells can be collected and frozen around 30 days or 45 days after initiation of the differentiation protocol (the exact timing of collection is PSC-line dependent and should be experimentally defined based on the day of maximum yield). The yield is usually between 25 and 120 myeloid cells for every starting PSC, and their post-thaw viability is 85±10%.

[00152] Furthermore, this also provides methods to generate “matured” myeloid progenitor cells. Maturation of the iPSC-derived myeloid progenitor cells is advantageous to imbue these therapeutic cells with an array of enhanced functions. For example, the maturation process can offer the cells resistance to M2 polarization, which typically involves a shift toward tumor-promoting phenotypes, enhancing their potency in cancer-fighting applications. Moreover, matured myeloid progenitor cells exhibit an improved recovery rate post-cryopreservation, conferring robust stability and longevity to these cells. The maturation process can increase cell viability, increasing recovery rate post-cryopreservation by increasing pro-survival signaling molecules like c-FLIP (cFLAR), MCL-1 and A1 (Bfl-1). The matured cells can also be characterized by enhanced migration capacity, facilitating their efficient navigation towards tumors. As a result of maturation, the cells may harness an elevated ability to phagocytose tumor cells, coupled with an improved chemokine and cytokine expression, which exemplifies their enhanced therapeutic utility. The described maturation process, thus, strengthens these cells, rendering them a powerful asset in the field of cancer immunotherapy.

[00153] In some embodiments the present disclosure provides methods for generating myeloid cells from pluripotent stem cells. In some such embodiments the pluripotent stem cells are from any mammalian species, but preferably from humans. In some embodiments the pluripotent stem cells are either induced pluripotent stem cells (“iPS cells” or “iPSCs”), or embryonic stem cells (“ES cells” or “ESCs”). Such methods involve a step where the pluripotent stem cells are cultured under conditions that induce myeloid differentiation, leading to the generation of CD45⁺/CD14⁺/CX3CR1⁺ myeloid cells. In some such embodiments these cells express Lyz2, CD14, TGM2, SLAMF7, IL1-Rn, GPR34 when evaluated by qPCR. In some embodiments the differentiation medium comprises BMP4, GM-CSF, VEGF, SCF, IL3, TPO, M-CSF and FLT31. In some embodiments the medium further optionally comprises bFGF.

[00154] In some of the embodiments, the pluripotent stem cells are cultured under conditions that induce myeloid differentiation, leading to the generation of CX3CR1⁺

myeloid cells. In some of the embodiments, the pluripotent stem cells are cultured under conditions that induce myeloid differentiation, leading to the generation of CD45⁺ myeloid cells. In some embodiments the pluripotent stem cells are cultured or expanded in a bioreactor. In some embodiments the pluripotent stem cells are cultured in a cell factory under active gassing. By “active gassing” is meant exerting or applying a gas mixture pressure gradient in the cell factory or cell factories. Gas mixtures contemplated by the present disclosure include ratios of about 1% to about 20% CO₂ to about 80% to about 99% air, about 3% CO₂ to about 97% air and about 5% CO₂ to about 95% air.

[00155] In some embodiments, the myeloid cells are cultured from about 1 to about 30 days, from about 2 to about 25 days, from about 2 to about 20 days, from about 2 to about 18 days, from about 2 to about 15 days, from about 2 to about 10 days, from about 2 to about 8 days, from about 2 to about 6 days or from about 2 to about 4 days. In some embodiments, the myeloid cells are at least 90% CD45⁺, at least 91% CD45⁺, at least 92% CD45⁺, at least 93% CD45⁺, at least 94% CD45⁺, or at least 95% CD45⁺. In some embodiments, the myeloid cells are cultured for about 2 days, for about 4 days, for about 6 days or about 8 days, for about 10 days, for about 12 days for about 14 days, for about 16 days, for about 18 days, for about 20 days, for about 22 days, for about 24 days, for about 26 days, for about 28 days or for about 30 days. In some embodiments, the myeloid cells are at least about 70% CD45⁺/CD14⁺/CX3CR1⁺, at least about 71% CD45⁺/CD14⁺/CX3CR1⁺, at least about 72% CD45⁺/CD14⁺/CX3CR1⁺, at least about 73% CD45⁺/CD14⁺/CX3CR1⁺, at least about 74% CD45⁺/CD14⁺/CX3CR1⁺, at least about 75% CD45⁺/CD14⁺/CX3CR1⁺, at least about 80% CD45⁺/CD14⁺/CX3CR1⁺, or at least about 85% CD45⁺/CD14⁺/CX3CR1⁺.

[00156] In some of the embodiments of the present disclosure that involve culturing pluripotent stem cells under conditions that induce myeloid differentiation (leading to the generation of CD45⁺/CD14⁺ /CX3CR1⁺ myeloid cells), a multi-step process is used in which the cells are cultured with different combinations of cytokines and tissue culture media at each stage. Steps in these multi-step processes may result in inducing differentiation of pluripotent stem cells into primitive hemangioblasts, and/or inducing differentiation of primitive hemangioblasts into myeloid progenitors.

[00157] In some embodiments, the methods provided herein for the generation of CD45⁺/CD14⁺ /CX3CR1⁺ myeloid cells from pluripotent stem cells comprise performing one or more of the following steps: First, contacting a cell culture with a first composition

comprising BMP4 in a culture medium, wherein when the cell culture is initially contacted with the first composition the cell culture comprises pluripotent stem cells. A small molecule able to activate the same pathway as BMP4 can be used; Second, contacting the cell culture with a second composition comprising one or more of SCF, and VEGF, and optionally bFGF, (for example each of SCF, and VEGF, with or without bFGF) in a hematopoietic cell medium; Third, contacting the cell culture with a third composition comprising one or more of SCF, IL-3, TPO, M-CSF, and FLT3 ligand (for example each of SCF, IL-3, TPO, M-CSF, and FLT3 ligand) in a hematopoietic cell medium; and fourth, contacting the cell culture with a fourth composition comprising one or more of M-CSF, FLT3 ligand, and GM-CSF (for example each of M-CSF, FLT3 ligand, and GM-CSF) in a hematopoietic cell medium. In some embodiments all of the above four steps are performed in order. In some of such embodiments the medium used for any of these four steps is a serum free medium. In some of such embodiments the medium used for any of these four steps is a chemically defined medium.

[00158] In the first of the above four steps, in some embodiments a tissue culture medium suitable for maintenance of stem cells is used, while in other embodiments a tissue culture medium suitable for differentiation of stem cells is used. In the last three of the above four steps, any suitable hematopoietic cell medium can be used.

[00159] In some embodiments, when carrying out the methods described above or elsewhere herein for the generation of myeloid cells from pluripotent stem cells, instead of discarding the tissue culture supernatant when performing media changes, the supernatant is centrifuged, and the cells present in the supernatant are recovered and added back to the cell cultures. This is advantageous because certain of the cell types induced during the conversion of pluripotent stem cells to myeloid cells are found predominantly in the cell supernatants — as opposed to being in the layer of cells that adheres to the cell culture plates. Thus, in some embodiments, when media is changed cells present in the culture supernatant are recovered and added back to the cell cultures. In some embodiments, for media exchanges performed when the cells are in contact with the third composition or the fourth composition, cells present in the culture supernatant are recovered and added back to the cell cultures. In some embodiments the present disclosure provides myeloid cells or microglial progenitor cells, such as those produced by the methods described herein. In some embodiments the present disclosure provides a “substantially pure” populations of such cells.

[00160] For example, in some embodiments during differentiation, myeloid progenitor cells begin to emerge as “floaters”. For example, in some embodiments, differentiation of cells into myeloid progenitor cells involves adherent cell culture. However, during differentiation, progenitor cells release from tissue culture dishes in which the cells are cultured and float or suspend in the tissue culture medium. In some embodiments, the progenitor cells begin to emerge as floaters around day 8 of differentiation. In some embodiments, the progenitor cells begin to emerge as floaters around day 9 of differentiation. In some embodiments, the progenitor cells begin to emerge as floaters around day 10 of differentiation.

[00161] Accordingly, in some embodiments, prior to day 10, progenitor cells started to emerge as floaters. Accordingly, on Day 10 floating progenitor cells can be collected from the supernatant fraction, pelleted, and resuspended in culture medium (*e.g.*, StemPro-34 SFM medium containing Flt-3, M-CSF, and GM-CSF. In some embodiments, the cells can be further cultured until at about day 30 or day 45, at which point, the generated cells (*i.e.*, myeloid progenitor cells).

[00162] In some embodiments, myeloid progenitor cells are subjected to a maturation phase. The maturation of the myeloid progenitor cells can be advantageous as maturation can imbue the myeloid progenitor cells with added functionality. For example, the maturation phase can offer the cells resistance to M2 polarization, which typically involves a shift toward tumor-promoting phenotypes, enhancing their potency in cancer-fighting applications. Moreover, matured myeloid progenitor exhibit an improved recovery rate post-cryopreservation, conferring robust stability and longevity to the myeloid progenitor cells by increasing expression of pro-survival signaling molecules like cFLAR, A1, MCL-1. They are also characterized by enhanced migration capacity, facilitating their efficient navigation towards tumors. Their elevated ability to phagocytose tumor cells, coupled with their improved cytokine expression, exemplifies an enhanced therapeutic utility. The described maturation process, thus, strengthens these cells, rendering them a powerful asset in the field of cancer immunotherapy.

[00163] In some embodiments, to initiate maturation, floating progenitor cells are harvested, *e.g.*, harvested on either day 30 or day 45, from the supernatant fraction as described. These cells are then centrifuged to form a pellet and subsequently resuspended in a specially tailored maturation culture medium. In some embodiments, the maturation medium

is an XVIVO-15 culture blend enriched with GlutaMax and M-CSF. In some embodiments, the prepared cells are then seeded onto plastic dishes for cultivation. In some embodiments, the myeloid progenitor cells are matured for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 days. For example, in some embodiments, the myeloid progenitor cells are matured in maturation medium for a duration of 7 days. Post-maturation, the myeloid progenitor cells can be evaluated for the expression of XCR1, CD66B, and CD11b, to confirm maturation. In particular, matured myeloid progenitor cells can be identified based on their unique expression pattern: a lack of XCR1 and CD66B expression combined with the presence of CD11b expression. In some embodiments myeloid progenitor cells expressed LYZ2, MMP7, CD14, SLAMF7, TGM2, IL-1Rn, and GPR34.

III. Compositions

[00164] Certain aspects of the disclosure provide compositions having any of the populations of engineered cells described herein. In some embodiments, the composition is a pharmaceutical composition that includes a population of engineered cells that a cytokine, and a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the cytokine is TNF α , IL-1RA, IL-1 β , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-20, IL-21, IL-23, IL-33, IL-37, TRAIL, MIF, TGF β , IFN α , IFN γ , or GM-CSF. In some embodiments, the cytokine is IL-12.

[00165] In some embodiments, the composition (*e.g.*, pharmaceutical composition) includes a population of engineered immune cells that secrete the cytokine (*e.g.*, IL-12). In some embodiments, the population of engineered immune cells secrete the cytokine at between 1 and 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of 1 million cells per 24 hours. In some embodiments, the population of engineered immune cells secrete the cytokine at a concentration that is below 1,000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete the cytokine at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete cytokine at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete the cytokine at below 1ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells includes one or more of myeloid progenitor cells, microglia precursor cells, microglial cells, T cells, natural killer cells, or macrophages.

[00166] In other embodiments, the composition (*e.g.*, pharmaceutical composition) includes a population of engineered myeloid progenitor cells that secrete the cytokine (*e.g.*, IL-12). In some embodiments, the population of engineered myeloid progenitor cells has a genetic modification to elicit or enhance an amount of the cytokine that is secreted by the engineered myeloid progenitor cells as compared to myeloid progenitor cells not comprising the genetic modification. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 1 and 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of 1 million cells per milliliter for 24 hrs. In some embodiments, the engineered myeloid progenitor cells secrete the cytokine at a concentration that is below 500 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at below 1ng/mL/1M cells/24hr.

[00167] In some embodiments, the composition (*e.g.*, pharmaceutical composition) includes a population of engineered immune cells that secrete IL-12. In some embodiments, the population of engineered immune cells secrete IL-12 at between 1 and 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of 1 million cells per 24 hours. In some embodiments, the population of engineered immune cells secrete IL-12 at a concentration that is below 1,000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at below 1ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells includes one or more of myeloid progenitor cells, microglia precursor cells, microglial cells, T cells, natural killer cells, or macrophages.

[00168] In other embodiments, the composition (*e.g.*, pharmaceutical composition) includes a population of engineered myeloid progenitor cells that secrete IL-12. In some embodiments, the population of engineered myeloid progenitor cells has a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the engineered myeloid progenitor cells as compared to myeloid progenitor cells not comprising the genetic modification. In some embodiments, the population of engineered myeloid progenitor cells

secrete IL-12 at between 1 and 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of 1 million cells per milliliter for 24 hrs. In some embodiments, the engineered myeloid progenitor cells secrete IL-12 at a concentration that is below 500 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at below 1ng/mL/1M cells/24hr.

[00169] In some embodiments, a composition (*e.g.*, pharmaceutical composition) described herein includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that secrete IL-12 having an amino acid sequence as set forth in SEQ ID NO: 1. In some embodiments, the IL-12 secreted by the population of engineered cells in the composition has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or is identical to the amino acid sequence set forth in SEQ ID NO: 1. In some embodiments, the IL-12 is a human IL-12. In some embodiments, the IL-12 is an engineered or variant IL-12.

[00170] In some embodiments, a composition (*e.g.*, pharmaceutical composition) described herein includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that has a genetic modification to elicit or enhance an amount of the cytokine (*e.g.*, IL-12) that is secreted by the cells. In some embodiments, the genetic modification enhances the expression, stability or secretion of the cytokine. In some embodiments, the genetic modification includes an integration of a heterologous nucleic acid encoding cytokine into the genome of the cells. In some embodiments, the cytokine is IL-12.

[00171] In some embodiments, a composition (*e.g.*, pharmaceutical composition) described herein includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) expresses one or more additional cytokines.

[00172] In some embodiments a composition (*e.g.*, pharmaceutical composition) described herein includes a population of engineered cells (*e.g.*, a population of engineered immune

cells or a population of engineered myeloid progenitor cells) is able to present antigens to T cells. In some embodiments, the T cells are CD8⁺ T cells. In other embodiments, the T cells are CD4⁺ T cells. In some embodiments, the T cells are modified T cells. In some embodiments, the modified T cells are CAR-T cells. In some embodiments, the presentation of tumor antigens by the population of engineered cells results in activation of the T cells in a subject that has been administered the composition. In some embodiments, the presentation of tumor antigens by the population of engineered cells results in production of tumor antigen-specific T cells in a subject that has been administered the composition.

[00173] In some embodiments, a composition (*e.g.*, pharmaceutical composition) described herein includes a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that can activate or enhance an immune response when administered to a subject. In some embodiments, the population of engineered cells results in the activation or enhancement of an immune response to an antigen (*e.g.*, a tumor antigen) in a subject that has been administered the composition. In some embodiments, the population of engineered cells results in the activation or enhancement of a T-cell mediated immune response to an antigen in a subject that has been administered the composition. In some embodiments, the T cell-mediated immune response is a CD8⁺ T cell-mediated immune response. In other embodiments, the T cell-mediated immune response is a CD4⁺ T cell-mediated immune response. In some embodiments, the subject has a tumor, and the antigen is a tumor antigen.

[00174] In some embodiments, a composition (*e.g.*, pharmaceutical composition) described herein includes population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that can further express cell surface proteins that enhance presentation of antigen (*e.g.*, tumor antigen) to T cells and/or the immune triggered by interacting with the T cells.

[00175] In some embodiments, each of the engineered cells in the populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) included in the composition (*e.g.*, pharmaceutical composition) described herein has a major histocompatibility complex (MHC) molecule on the cell surface. In some embodiments, the MHC molecule binds to and presents an antigen to T cells. In some embodiments, the MHC molecule is an MHC class I molecule or an MHC class II molecule. In some embodiments, each of the engineered cells overexpress the MHC molecule

on the cell surface. In some embodiments, the overexpression of the MHC molecule on the cell surface increases the presentation of the antigens to T cells.

[00176] In some embodiments, each of the engineered cells in the populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) included in the composition (*e.g.*, pharmaceutical composition) described herein has a co-stimulatory molecule on the cell surface. In some embodiments, the co-stimulatory molecule selected from CD2, CD7, B7-1 (CD80), B7-2 (CD86), 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD58, CD70, CD80, CD83, CD86, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3TR6, ILT3, ILT4, HVEM, BTLA, an agonist or antibody that binds a Toll ligand receptor, or B7-H3 ligand. In some embodiments, the said co-stimulatory molecule is CD80, CD86, or CD40. In some embodiments, the co-stimulatory molecule enhances the immune response triggered by the population of engineered cells. In some embodiments, the co-stimulatory molecule enhances a T cell-mediated immune response by interacting with T cells, *e.g.*, by interacting with a cell surface protein expressed by the T cells. In some embodiments, the T cell-mediated immune response is a CD8⁺ T cell mediated response. In other embodiments, the T cell-mediated immune response is a CD4⁺ T cell mediated response.

[00177] In some embodiments, each of the engineered cells in the populations of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) included in the composition (*e.g.*, pharmaceutical composition) described herein has a targeting moiety on the cell surface. In some embodiments, the targeting moiety binds to a protein expressed in the cell surface of T cells. In some embodiments, the targeting moiety binds to a protein expressed in the cell surface of CD8⁺ T cells. In other embodiments, the targeting moiety binds to a protein expressed in the cell surface of CD4⁺ T cells. In some embodiments, the targeting moiety is an engineered receptor. In some embodiments, the targeting moiety is a CAR.

[00178] The pharmaceutically acceptable excipient, carrier, or diluent can be any excipient, carrier or diluent known in the art. For instance, the pharmaceutically acceptable excipient, carrier, or diluent can be a cell culture medium (*e.g.*, one that optionally lacks any animal-derived component), sterilized water, physiological saline, general buffers (*e.g.*, phosphoric acid, citric acid, other organic acids, etc.), stabilizers, salts, anti-oxidants,

surfactants, suspensions, isotonic agents, and/or preservatives may be included in a pharmaceutical composition described herein. The specific excipient, carrier, or diluent will depend on the route of administration intended for the pharmaceutical composition. In some embodiments, the pharmaceutically acceptable excipient, carrier, or diluent is an excipient, carrier, or diluent suitable for treatment of a tumor in a subject. In some embodiments, the pharmaceutically acceptable excipient, carrier, or diluent is an excipient, carrier, or diluent suitable for administration of the pharmaceutical composition by injection to a subject. In some embodiments, the pharmaceutically acceptable excipient, carrier, or diluent is an excipient, carrier, or diluent suitable for administration of the composition by direct injection into the site of a tumor (*e.g.*, a brain tumor).

[00179] The compositions (*e.g.*, pharmaceutical compositions) described herein can be used for the treatment of a disease, such as cancer, in a subject. For instance, the pharmaceutical formulation can be used for the treatment of a tumor formed by cancerous cells. In some embodiments, the composition is for use in treating a subject having a tumor. The tumor can be, for example, anal cancer, bile duct cancer, bladder cancer, bone cancer, bowel cancer, brain tumor (*e.g.*, glioblastoma), breast cancer, cervical cancer, choriocarcinoma, colon cancer, colorectal cancer, endometrial cancer, eye cancer, gallbladder cancer, gastric cancer, gestational trophoblastic tumor (GTT), head and neck cancer, kidney cancer, laryngeal cancer, leukemia, liver cancer, lung cancer, mesothelioma, molar pregnancy, mouth and oropharyngeal cancer, nasal and sinus cancers, nasopharyngeal cancer, esophageal cancer, ovarian cancer, pancreatic cancer, penile cancer, prostate cancer, rare cancers, rectal cancer, salivary gland cancer, secondary cancers, skin cancer (non-melanoma), stomach cancer, testicular cancer, thyroid cancer, unknown primary cancer, uterine cancer, vaginal cancer, or vulval cancer. In some embodiments, the tumor is a leukemia, a carcinoid, a melanoma, a lymphoma, a myeloma, a carcinoid, a sarcoma, a carcinoma, an adenoma, an adenocarcinoma, or a cancer of unknown primary. The tumor can be metastatic or non-metastatic. In some embodiments, the composition is for use in treating a brain tumor. In some embodiments, the composition is for use in treating a glioblastoma.

Dosage forms

[00180] Certain aspects of the disclosure provide dosage forms of a composition (*e.g.*, a pharmaceutical composition) that includes a population of engineered cells (*e.g.*, a population

of engineered immune cells or a population of engineered myeloid cells) suitable for administration to a subject, including any of the compositions described herein. The dosage form can be in any form suitable for administration to a subject by any suitable route, including orally, pulmonarily, intranasally, parenterally (intravenously, intramuscularly, intraperitoneally, or subcutaneously), rectally, intralymphatically, intracranial, intracerebraor topically. In some embodiments, the dosage form is suitable for administration by injection into a subject.

Kits

[00181] Certain aspects of the disclosure provide for kits having a dosage form of a composition (*e.g.*, a pharmaceutical composition) that includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid cells) suitable for administration to a subject, including any of the compositions described herein.

[00182] In some embodiments, the kit includes instructional material for the use of said dosage form. In some embodiments, the instructional material includes instructions of preparing the composition or the population of engineered cells for administration into a subject. Such instructions can include, but are not limited to, instructions for preparing or storing the composition, diluting the population of engineered cells, adding additional additives to the treatment, or combining the composition with an additional anti-cancer treatment. In some embodiments, the instructional material includes instructions for administering the dosage form into a subject. For instance, the instructional material can include instructions for administering the dosage form by injection to the site of a tumor in a subject.

[00183] In some embodiments, the kit further includes an applicator for administering a composition, including a pharmaceutical composition, described here. The applicator can be any device suitable for administration of the pharmaceutical compositions described herein to a subject, including, but not limited to, a hypodermic syringe, a needle, a balloon-dilating catheter, a pipette, and the like. The applicator can also be a single-use or multiple-use administration device, and can be included in the kit as a pre-filled device with the pharmaceutical composition.

Devices

[00184] Certain aspects of the disclosure provide for devices for administering a therapy to a subject having any of the compositions (*e.g.*, pharmaceutical compositions) described herein. The device can be selected or modified depending on the route of administration of the composition. For instance, the device can be a device for administration by injection into a subject, *e.g.*, a hypodermic syringe. The device can also be an implantable device. In some embodiments, the device is a specialized device for delivery of cell therapies into a subject, such as those described in U.S. Patent No. 11,666,710 B2, which is incorporated by reference. In some embodiments, the device is pre-filled with the composition.

IV. Method of Treatment

[00185] One aspect of the present disclosure provides methods for treating a subject having a tumor, or for stimulating an immune response against a tumor in a subject. In some embodiments, the method is a method for stimulating an immune response against a tumor in a subject, the method including administering a composition that includes a population of engineered cells that secrete a cytokine (*e.g.*, IL-12) as described herein, and allowing the population of engineered immune cells to secrete the cytokine, thereby stimulating the immune response against the tumor. In other embodiments, the method is a method for treating a subject having a tumor, the method including administering a composition that includes a population of engineered cells that secrete a cytokine (*e.g.*, IL-12) as described herein, and allowing the population of engineered immune cells to secrete the cytokine, thereby treating the tumor in the subject. In some embodiments, the cytokine is TNF α , IL-1RA, IL-1 β , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-20, IL-21, IL-23, IL-33, IL-37, TRAIL, MIF, TGF β , IFN α , IFN γ , or GM-CSF. In some embodiments, the cytokine is IL-12.

[00186] In some embodiments, the method includes administering to a subject a composition (*e.g.*, a pharmaceutical composition) that includes a population of engineered immune cells that secrete the cytokine (*e.g.*, IL-12) as described herein. In some embodiments, the population of engineered immune cells secrete the cytokine at between 1 and 1,000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete the cytokine at a concentration that is below 1,000 ng/mL, as measured by

HTRF, when the cells are cultured at a density of 1 million cells per milliliter for 24 hrs. In some embodiments, the population of engineered immune cells secrete the cytokine at a concentration that is below 500 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete the cytokine at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete the cytokine at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete the cytokine at below 1ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells includes one or more of myeloid progenitor cells, microglia precursor cells, microglial cells, T cells, natural killer cells, or macrophages.

[00187] In other embodiments, the method includes administering to a subject a composition (*e.g.*, a pharmaceutical composition) that includes a population of engineered myeloid progenitor cells that secrete a cytokine (*e.g.*, IL-12) as described herein. In some embodiments, the population of engineered myeloid progenitor cells has a genetic modification to elicit or enhance an amount of the cytokine that is secreted by the engineered myeloid progenitor cells as compared to myeloid progenitor cells not comprising the genetic modification. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 1 and 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of 1 million cells per milliliter for 24 hrs. In some embodiments, the engineered myeloid progenitor cells secrete the cytokine at a concentration that is below 1000 ng/mL/1M cells/24hr. In some embodiments, the engineered myeloid progenitor cells secrete the cytokine at a concentration that is below 500 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete the cytokine at below 1ng/mL/1M cells/24hr.

[00188] In some embodiments, the method includes administering to a subject a composition (*e.g.*, a pharmaceutical composition) that includes a population of engineered immune cells that secrete IL-12 as described herein. In some embodiments, the population of engineered immune cells secrete IL-12 at between 1 and 1,000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at a concentration that is below 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of

1 million cells per milliliter for 24 hrs. In some embodiments, the population of engineered immune cells secrete IL-12 at a concentration that is below 500 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells secrete IL-12 at below 1ng/mL/1M cells/24hr. In some embodiments, the population of engineered immune cells includes one or more of myeloid progenitor cells, microglia precursor cells, microglial cells, T cells, natural killer cells, or macrophages.

[00189] In other embodiments, the method includes administering to a subject a composition (*e.g.*, a pharmaceutical composition) that includes a population of engineered myeloid progenitor cells that secrete IL-12 as described herein. In some embodiments, the population of engineered myeloid progenitor cells has a genetic modification to elicit or enhance an amount of IL-12 that is secreted by the engineered myeloid progenitor cells as compared to myeloid progenitor cells not comprising the genetic modification. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at between 1 and 1,000 ng/mL, as measured by HTRF, when the cells are cultured at a density of 1 million cells per milliliter for 24 hrs. In some embodiments, the engineered myeloid progenitor cells secrete IL-12 at a concentration that is below 1000 ng/mL/1M cells/24hr. In some embodiments, the engineered myeloid progenitor cells secrete IL-12 at a concentration that is below 500 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at between 2 and 1000 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at between 1 and 2 ng/mL/1M cells/24hr. In some embodiments, the population of engineered myeloid progenitor cells secrete IL-12 at below 1ng/mL/1M cells/24hr.

[00190] In some embodiments the method includes administering to a subject a pharmaceutical composition that includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that secrete IL-12 having an amino acid sequence as set forth in SEQ ID NO: 1. In some embodiments, the IL-12 secreted by the population of engineered cells in the pharmaceutical composition has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or is identical to the amino acid sequence set

forth in SEQ ID NO: 1. In some embodiments, the IL-12 is a human IL-12. In some embodiments, the IL-12 is an engineered or variant IL-12. In some embodiments, the population of engineered cells secretes IL-12 constitutively.

[00191] In some embodiments, the method includes administering to a subject a pharmaceutical composition that includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that has a genetic modification to elicit or enhance an amount of the cytokine (*e.g.*, IL-12) that is secreted by the cells. In some embodiments, the genetic modification enhances the expression, stability or secretion of the cytokine. In some embodiments, the genetic modification includes an integration of a heterologous nucleic acid encoding the cytokine into the genome of the cells. In some embodiments, the cytokine is IL-12.

[00192] In some embodiments, the method includes administering to a subject a pharmaceutical composition that includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) expresses one or more additional cytokines.

[00193] In some embodiments, the method includes administering a pharmaceutical composition that includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that is able to present antigens to T cells. In some embodiments, the T cells are CD8⁺ T cells. In other embodiments, the T cells are CD4⁺ T cells. In some embodiments, the T cells are modified T cells, *e.g.*, CAR-T cells. In some embodiments, the presentation of tumor antigens by the population of engineered cells results in activation of the T cells in the subject. In some embodiments, the presentation of tumor antigens by the population of engineered cells results in production of tumor antigen-specific T cells in the subject.

[00194] In some embodiments, the method includes administering a pharmaceutical composition that includes a population of engineered cells described herein (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) the stimulation of an immune response against a tumor antigen in the subject. In some embodiments, the immune response is a T-cell mediated immune response. In some embodiments, the T cell-mediated immune response is a CD8⁺ T cell-mediated immune response. In other embodiments, the T cell-mediated immune response is a CD4⁺ T cell-

mediated immune response. In some embodiments, the immune response comprises secretion of pro-inflammatory cytokines by T regs. In some embodiments, the pro-inflammatory cytokines comprise IFN γ , TNF α , or both IFN γ and TNF α .

[00195] In some embodiments, the method includes administering a pharmaceutical composition that includes population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that can further express cell surface proteins that enhance presentation of antigen (*e.g.*, tumor antigen) to T cells and/or the immune triggered by interacting with the T cells. In some embodiments, each of the engineered cells in the populations of engineered cells has a major histocompatibility complex (MHC) molecule on the cell surface. In some embodiments, the MHC molecule binds to and presents an antigen to T cells. In some embodiments, the MHC molecule is an MHC class I molecule or an MHC class II molecule. In some embodiments, each of the engineered cells overexpress the MHC molecule on the cell surface, thereby increasing the presentation of the antigens to T cells. In some embodiments, each of the engineered cells in the populations of engineered cells has a co-stimulatory molecule on the cell surface. In some embodiments, the co-stimulatory molecule selected from CD2, CD7, B7-1 (CD80), B7-2 (CD86), 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD58, CD70, CD80, CD83, CD86, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3TR6, ILT3, ILT4, HVEM, BTLA, an agonist or antibody that binds a Toll ligand receptor, or B7-H3 ligand. In some embodiments, the said co-stimulatory molecule is CD80, CD86, or CD40. In some embodiments, the co-stimulatory molecule enhances a T cell-mediated immune response (*e.g.*, a CD8⁺ T cell-mediated immune response) by interacting with T cells, *e.g.*, by interacting with a cell surface protein expressed by the T cells. In some embodiments, each of the engineered cells in the populations of engineered cells has a targeting moiety on the cell surface. In some embodiments, the targeting moiety binds to a protein expressed in the cell surface of T cells. In some embodiments, the targeting moiety is an engineered receptor, *e.g.*, a CAR.

[00196] In some embodiments, the method includes administering a pharmaceutical composition that includes a population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells) that is allogenic to the subject. In some embodiments, the method includes administering a pharmaceutical composition that includes a population of engineered cells (*e.g.*, a population of engineered

immune cells or a population of engineered myeloid progenitor cells) that is autologous to the subject.

[00197] In some embodiments, the stimulation of an immune response against the tumor results in the improvement of at least one symptom associated with the tumor, *e.g.*, as determined by responsiveness/non-responsiveness, or indicators known in the art and described herein. Non-limiting examples of the common medical technologies and methods used to examine and diagnose a tumor in a subject include, but are not limited to, X-rays, computed tomography (CT) scan, magnetic resonance imaging (MRI), positron emission tomography (PET), bone scintigraphy (Bone Scan), biopsy, EQ-5D questionnaire for assessing quality of life, PERCIST criteria for assessing disease progression, numeric rating scale for assessing pain degree. The choice of technologies and methods, and the time and frequency of examination can be determined and/or adjusted by a person skilled in the art based on the subject's specific conditions.

[00198] In some embodiments, the method further includes examining the subject for responsiveness to the pharmaceutical composition that includes the population of engineered cells (*e.g.*, a population of engineered immune cells or a population of engineered myeloid progenitor cells). The responsiveness of the subject can be determined as an improvement of at least one parameter of disease progression (*e.g.*, tumor progression). The subject can have partial response or complete response to a treatment. The response to a treatment can be determined based on methods known in the art. A person skilled in the art can determine the proper methods based on the type of diseases being evaluated. Non-limiting examples of the methods include RECIST criteria, ICDS criteria and PERCIST criteria.

Subjects

[00199] Certain aspects of the methods described herein relate to subjects having a tumor. In some embodiments, subject has a tumor selected from anal cancer, bile duct cancer, bladder cancer, bone cancer, bowel cancer, brain tumor (*e.g.*, glioblastoma), breast cancer, cervical cancer, choriocarcinoma, colon cancer, colorectal cancer, endometrial cancer, eye cancer, gallbladder cancer, gastric cancer, gestational trophoblastic tumor (GTT), head and neck cancer, kidney cancer, laryngeal cancer, leukemia, liver cancer, lung cancer, mesothelioma, molar pregnancy, mouth and oropharyngeal cancer, nasal and sinus cancers, nasopharyngeal cancer, esophageal cancer, ovarian cancer, pancreatic cancer, penile cancer,

prostate cancer, rare cancers, rectal cancer, salivary gland cancer, secondary cancers, skin cancer (non-melanoma), stomach cancer, testicular cancer, thyroid cancer, unknown primary cancer, uterine cancer, vaginal cancer, or vulval cancer. In some embodiments, the subject has a leukemia, a carcinoid, a melanoma, a lymphoma, a myeloma, a carcinoid, a sarcoma, a carcinoma, an adenoma, an adenocarcinoma, or a cancer of unknown primary. In some embodiments, the tumor is metastatic or non-metastatic. In some embodiments, the tumor is a brain tumor. In some embodiments, the brain tumor is a glioblastoma. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human.

Methods of administration

[00200] The composition (*e.g.*, a pharmaceutical composition) of the methods described herein can be administered to a subject by any suitable route of administration. Methods of administration of pharmaceutical compositions described herein are known to those in the art. In some embodiments, the method includes administering the composition that includes a population of engineered cells described herein orally, pulmonarily, intranasally, parenterally (intravenously, intramuscularly, intraperitoneally, or subcutaneously), rectally, intralymphatically, or topically. In some embodiments, the composition is administered intratumorally. In some embodiments, the composition is administered by direct injection into the site of the tumor.

[00201] The composition can be administered to the subject at one time or over a series of administrations and may be administered to the patient at any time from diagnosis of the tumor onwards. For instance, the composition can be administered as one or more doses over the course of a treatment. The doses can be administered using the same or a different route of administration, and can contain the same amount or a different amount of the composition.

[00202] In some embodiments, a dose of the composition is administered about once every day, about once every 2 days, about once every 3 days, about once every 4 days, about once every 5 days, about once every 6 days, about once every week, about once every 8 days, about once every 9 days, about once every 10 days, about once every 11 days, about once every 12 days, about once every 13 days, about once every 2 weeks, about once every 15 days, about once every 16 days, about once every 17 days, about once every 18 days, about once every 19 days, about once every 20 days, about once every 3 weeks, about once every 22 days, about once every 23 days, about once every 24 days, about once every 25 days,

about once every 26 days, about once every 27 days, about once every 4 weeks, about once every 29 days, about once every 30 days, about once every 31 days, about once every 32 days, about once every 33 days, about once every 34 days, about once every 5 weeks, about once every 36 days, about once every 37 days, about once every 38 days, about once every 39 days, about once every 40 days, or about once every 41 days, about once every 6 weeks, about once every 7 weeks, about once every 8 weeks, about once every 9 weeks, about once every 10 weeks, about once every 11 weeks, about once every 12 weeks, about once every 13 weeks, about once every 15 weeks, about once every 16 weeks, about once every 17 weeks, about once every 18 weeks, about once every 19 weeks, about once every 20 weeks, about once every 21 weeks, about once every 22 weeks, about once every 23 weeks, about once every 24 weeks or 6 months, or about once every more than 24 weeks or 6 months. Such doses can be for a specified period of time. For example, the dose can be for about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 15 weeks, about 18 weeks, about 22 weeks, about 24 weeks or six months, about 7 months, about 8 months about 9 months, about 10 months, about 11 months, about 12 months or more than 12 months.

[00203] The duration of administration can depend on the route of administration of the composition. In some embodiments, the composition is administered to a subject for over less than 5 minutes, about 5 minutes, about 10 minutes, about 15 minutes about 20 minutes, about 25 minutes, about 30 minutes, about 40 minutes, about 50 minutes, about 60 minutes, about 70 minutes about 80 minutes, about 90 minutes, about 100 minutes, about 110 minutes, about 120 minutes, about 150 minutes, about 180 minutes, or more than 180 minutes.

Additional anti-cancer therapies

[00204] In some embodiments, the methods describe herein include administering an additional anti-cancer therapy to the subject. The additional anti-cancer therapy can be administered before, after or simultaneous to the pharmaceutical composition that includes the population of engineered cells (*e.g.*, the population of engineered immune cells or the population of engineered myeloid progenitor cells). In some embodiments, the additional anti-cancer therapy includes a hormone blocking therapy, chemotherapy, an immune checkpoint inhibitor, a kinase inhibitor, a therapeutic antibody or binding fragments thereof, electromagnetic field therapy, a cell therapy, radiation therapy, surgery (*e.g.*, resection surgery), or any combination thereof. Other exemplary additional anti-cancer therapies are

described in, for example, Bernhard, C., et al., (2023) Int J Mol Sci. 24(11):9137, and Wang, M., et al. (2023) Front Immunol. 14:1175118.

[00205] In some embodiments, the additional anti-cancer therapy includes a hormone blocking therapy. Exemplary hormone blocking therapies include, but are not limited to, tamoxifen, anastrozole, and letrozole. In some embodiments, the additional anti-cancer therapy includes a chemotherapy. of such chemotherapies include platinum-based chemotherapy drugs (*e.g.*, cisplatin, carboplatin); electrochemotherapy; alkylating agents; taxanes (*e.g.*, paclitaxel (Taxol®), docetaxel (Taxotere®), EndoTAG-PM (a formulation of paclitaxel encapsulated in positively charged lipid-based complexes; MediGene), Abraxane® (a formulation of paclitaxel bound to albumin)); tyrosine kinase inhibitors (*e.g.*, imatinib/Gleevec®, sunitinib/Sutent®, dasatinib/Sprycel®); antimetabolites, such as 5-fluorouracil (5- FU), 6-mercaptopurine (6-MP), Capecitabine (Xeloda®), Cladribine, Clofarabine, Cytarabine (Ara-C®), Floxuridine, Fludarabine, Gemcitabine (Gemzar®), Hydroxyurea, Methotrexate, Pemetrexed (Alimta®), Pentostatin, or Thioguanine; and combinations thereof.

[00206] In some embodiments, the additional anti-cancer therapy includes a kinase inhibitor. Exemplary kinase inhibitors include, but are not limited to Imatinib mesylate (approved for chronic myelogenous leukemia, gastrointestinal stromal tumor and some other types of cancer), Gefitinib (Iressa, also known as ZD1839), Erlotinib (marketed as Tarceva), Sorafenib, Sunitinib (Sutent), Dasatinib (Srycel), Lapatinib (Tykerb), Nilotinib (Tasigna), Bortezomib (Velcade); Janus kinase inhibitors, ALK inhibitors, crizotinib; Bcl-2 inhibitors, obatoclax, navitoclax, gossypol, PARP inhibitors, Iniparib, Olaparib, PBK inhibitors, perifosine, Apatinib, VEGF Receptor 2 inhibitors, AN-152, Braf inhibitors, vemurafenib, dabrafenib, LGX818, MEK inhibitors, trametinib, MEK162, CDK inhibitors, PD-0332991, Hsp90 inhibitors, salinomycin, or MCL-1 inhibitors. The kinase inhibitor can also include serine/threonine kinase inhibitors. Without being limiting, examples include Temsirolimus (Torisel), Everolimus (Afinitor), Vemurafenib (Zelboraf), Trametinib (Mekinist) or Dabrafenib (Tafinlar).

[00207] In some embodiments, the additional anti-cancer therapy includes an immune checkpoint inhibitor. An “immune checkpoint” refers to inhibitory pathways hardwired into the immune system that are crucial for maintaining self-tolerance and modulating the immune responses in peripheral tissues in order to minimize collateral tissue damage. Immune

checkpoint molecules can be stimulatory or inhibitory to an immune checkpoint. An immune checkpoint inhibitor inhibits inhibitory immune checkpoint molecules. In some embodiments, the immune checkpoint inhibitor includes an inhibitor of one or more immune checkpoint molecules selected from PD-1, PD-L1, CTLA-4, VISTA, PD-L2, IDO, ARG1, B7-H3, B7-H4, LAG3, 2B4, BTLA, TIM3, A2aR, KIR, IL4i1, VEGF, LILRB2, and LILRB4. In some embodiments, the immune checkpoint inhibitor is an antibody that binds an immune checkpoint molecule selected from PD-1, PD-L1, CTLA-4, VISTA, PD-L2, IDO, ARG1, B7-H3, B7-H4, LAG3, 2B4, BTLA, TIM3, A2aR, KIR, IL4i1, VEGF, LILRB2, and LILRB4.

[00208] In some embodiments, the additional anti-cancer therapy includes a therapeutic antibody or binding fragment thereof. The therapeutic antibody or binding fragment thereof can be an agonistic antibody (*i.e.*, activates a target protein) or an antagonistic antibody (*i.e.*, inhibits a target protein). Exemplary antibody or binding fragments thereof include, but are not limited, anti-EGFR antibodies, anti-VEGF antibodies, such as bevacizumab (Avastatin), anti-ErbB2 antibodies, such as trastuzumab and pertuzumab, as well as antibodies that bind and inhibit immune checkpoint molecules, such as anti-PD-1 antibodies, anti-PD-L1 antibodies, and anti-CTLA-4 antibodies.

[00209] In some embodiments, the additional anti-cancer therapy includes a cell therapy. In some embodiments, the cell therapy is a modified T cell, *e.g.*, CAR-T cell, therapy.

[00210] In some embodiments, the additional anti-cancer therapy includes a CD28 agonist. In some embodiments, the CD28 agonist is an anti-CD28 antibody.

[00211] In other embodiments, the additional anti-cancer therapy does not comprise a CD28 agonist. In some embodiments, the CD28 agonist is an anti-CD28 antibody.

[00212] In some embodiments, the additional anti-cancer therapy includes an electric field therapy. In some embodiments, the electric field therapy is an alternating electric field therapy (*e.g.*, a tumor treating field or TTField).

EXAMPLES

[00213] In order that this disclosure may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the disclosure in any manner.

Example 1: Methods for the generation of myeloid progenitor cells from human induced pluripotent stem cells (iPSCs)

[00214] The following example describes methods to generate myeloid progenitor cells from human induced pluripotent stem cells (iPSCs). These methods, enabled by the unique properties of stem cells to self-renew and their potential to differentiate into any cell type, allow for a more reliable and scalable production of myeloid progenitor cells as compared to existing autologous cell therapies. Furthermore, the methods described include certain “maturation” steps that endow iPSC-derived myeloid progenitor cells with additional functions, for example, an improved recovery rate following cryopreservation and a more suitable profile for anti-tumor therapy, for example, by expressing low levels of certain immune modulatory molecules, such as, IL-6, relative to bone marrow monocyte derived myeloid cells.

Myeloid progenitor cell differentiation

[00215] To generate myeloid progenitor cells, iPSCs were plated in tissue culture dishes with Essential 8 medium containing Y-27632 and cultured for 1 day, before cultures were changed to Essential 8 medium (without Y-27632) and cultured for additional 2 days with daily medium change to allow expansion. Then, cultures were changed to Essential 6 medium containing BMP-4 and cultured for 4 days with daily media change to start differentiation. After this period, the cultures were changed to StemPro-34 SFM medium containing GlutaMAX supplemented with SCF, VEGF, and bFGF for 2 days with daily medium changes. From day 6 to day 12, the culture media was replaced every 2 days with StemPro-34 SFM medium containing SCF, IL-3, M-CSF, Flt3 ligand and TPO. Beginning on day 14, cells in the supernatant fraction were re-inoculated into the culture every 2 or 3 days, along with fresh StemPro-34 SFM medium containing Flt-3, M-CSF and GM-CSF until approximately day 30, at which point, the generated cells in the culture supernatant (*i.e.*, myeloid progenitor cells) were subjected to a maturation phase, which imparts the myeloid progenitor cells with added functionality.

[00216] To mature the cells, the floating progenitor cells harvested on approximately day 30 were gathered from the supernatant fraction of the differentiation culture dishes, transferred to cell culture dishes and cultured for 7 days in maturation medium containing

XVIVO-15 culture blend supplemented with GlutaMax and M-CSF. Post-maturation, the cells underwent evaluation for the expression of XCR1, CD66B, and CD11b. Identification of the matured myeloid progenitor cells was achieved based on their unique expression pattern: a lack of XCR1 and CD66B expression combined with the presence of CD11b expression. Furthermore, following maturation, an improved rate of recovery cryopreservation was observed in the matured myeloid progenitor cells as compared to myeloid progenitor cells that were not subjected to the maturation step.

[00217] **FIG. 1** illustrates an exemplary cell differentiation scheme employed to generate myeloid progenitor cells from iPSCs. As detailed here, iPSCs were differentiated into myeloid progenitor cells within a span of 30 to 45 days following induction. The progenitor cells underwent a subsequent maturation process to acquire certain desired cellular characteristics, after which they were subjected to cryopreservation to maintain viability during storage.

Cryopreservation and thawing of myeloid progenitor cells

[00218] For cryopreservation, the cells were isolated and counted with an automated cell counter prior to freezing. Cells were resuspended in freezing medium and transferred to cryogenic vials. Cryogenic vials with cells were frozen with a controlled rate freezer and stored in liquid nitrogen vapor. At time of use, cells from the cryogenic vial were transferred to a centrifuge tube resuspended in cell-culture medium in a drop-wise fashion. Cells were centrifuged and resuspended in the appropriate assay or culture medium prior to utilization in experiments.

Example 2: Methods to genetically engineer iPSCs for the stable and sustained expression of IL-12

[00219] This example describes methods used to genetically engineer iPSCs for the stable and sustained expression of a cytokine transgene. In particular, this example describes experimental work that was conducted to integrate heterologous nucleic acids encoding IL-12 transgenes into the GAPDH STEL site of iPSCs. The resulting iPSCs, genetically engineered to drive increased IL-12 expression, were then subjected to a myeloid progenitor cell differentiation process as outlined in Example 1.

[00220] FIG. 2 is an illustration of the cloning strategy used to integrate IL-12 transgenes into the GAPDH STEL site. The IL-12 transgenes were integrated into the GAPDH STEL site by using a 2A sequence replacing the GAPDH stop codon to integrate the IL-12 transgene cassette into the GAPDH 3' UTR. The IL-12 transgenes were integrated using CRISPR-based gene editing systems as described below.

[00221] Various IL-12 cassettes were employed to genetically engineer the iPSCs. For example, a first cassette contained IL-12 (SEQ ID NO: 1:

MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEED
GITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWS
TDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTISTDLTFSVKSSRGSSDPQGVTC
GAATLSAERVVRGDNKEYEYSVEQCQEDSACPAAEESLPIEVMVDAVHKLKYENYTSS
FFIRDIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKRE
KKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSVPGVGPVGAR
NLPVATPDPGMFPC LHHSQNLLRAVSNMLQKARQTLEFYPTSEEIDHEDITKDKTS
TVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEF
KTMNAKLLMDPKRQIFLDQNM L AVIDELMQALNFNSETVPQKSSLEEDFYKTKIKL
CILLHAFRIRAVTIDRVMSYLNAS*). A second cassette contained IL-12 (SEQ ID NO: 1)
in combination with a truncated CD19 ("CD19t") (referred to herein as CD19t-IL-12; SEQ
ID NO: 2:

MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLKGTSDGPTQQLTWSRESP
LKPFLKLSLGLPGLGIHMRPLAIWLFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVN
VEGSGELFRWNVSDLGGLGCGLKNSRSEGPSSPSGKLMSPKLYVWAKDRPEIWEGE
PPCVPPRDSL NQSLSQDLTMAPGSTLWLSCGVPPDSVSRGPLSWTHVHPKGPKSLLS
LELKDDRPARDMWV METGLLLPRATAQDAGKYYCHRGNL TMSFHLEITARPVLWH
WLLRTGGWKVSAVTLAYLIFCLCSLVGILHLQRALVLRRKR).

[00222] The IL-12 cassettes were integrated using the CRISPR gene editing system. To achieve engineering, Cas RNPs were pre-complexed with guide RNAs specific to the GAPDH locus. The resulting protein complexes were electroporated together with a homology-directed DNA repair (HDR) template, containing either IL-12 or CD19t-IL-12 into the iPSCs. Cells having the desired genetic modifications were selected and expanded using methods described herein. In some instances, the IL-12 only cassette was integrated into two

alleles (biallelic) of GAPDH. In some instances, the IL-12 only cassette was integrated into a single allele (monoallelic) of GAPDH.

[00223] Prior to integrating the IL-12 cassettes into the genome of iPSCs, an analysis was conducted to identify an optimal integration site. For this analysis, the consideration of IL-12 transgenes exhibiting stable expression during extended cell culture (*e.g.*, > 5 cell divisions) and differentiation into myeloid progenitor cells was important. As a result, and by leveraging previously described work (see, WO2021072329A1, incorporated by reference), the GAPDH STEL site was selected.

[00224] Following integration, the payload candidate's expression cassette was governed by an endogenous gene promoter, *i.e.*, GAPDH. Consequently, the payload candidate's expression was tied to the endogenous gene's expression. The continued activity of the gene in the engineered cells implied that the linked payload transgene's expression would remain sustained and constitutive.

[00225] The successful integration of the IL-12 cassette into the cells was validated through a comprehensive series of polymerase chain reaction (PCR) screenings. Traditional PCR techniques served as the initial confirmation method, demonstrating the presence of the cassette within the genomic framework. In addition, droplet digital PCR (ddPCR) analyses were conducted to ascertain the copy number, thereby providing quantitative data as to the extent of integration. In addition, integration was further confirmed by Sanger sequencing. This multi-tiered approach to confirm successful integration ensured the reliability and success of the transgene integration process. Furthermore, production of IL-12 from the iPSCs genetically engineered was evaluated (data not shown) using a Förster Resonance Energy Transfer (FRET) cytokine assay, as described below. The production of IL-12 was determined to be about 1-2 ng (CD19t-IL-12), about 500 ng (IL-12 monoallelic), and about 100 ng (IL-12 biallelic) per mL per million cells per 24 hours.

Example 3: Characterization of iPSC-derived myeloid progenitor cells genetically engineered with an IL-12 transgene

[00226] This example describes the characterization of iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12. As described herein, iPSCs were genetically engineered to express IL-12 from the GAPDH locus and subsequently directed along a

specific differentiation pathway to produce genetically engineered myeloid progenitor cells. After differentiation, the genetically engineered myeloid progenitor cells were subjected to the maturation process described in Example 1. Subsequently, the genetically engineered myeloid progenitor cells underwent a thorough characterization process. This process was aimed at evaluating cellular features, determining their cell capabilities, and ensuring the cells possessed expected functionality arising from the genetic modifications and the subsequent differentiation and maturation stages.

Maturation & phenotype evaluated by flow cytometry

[00227] First, myeloid progenitor cells were characterized by fluorescence-activated cell sorting (FACS) analysis, a technique that involves determining and characterizing cell phenotype based on the expression of fluorescent markers. This method is useful for assessing phenotypic profiles of cells, giving important insights into their specific attributes and further validating the transformation and maturation process. FACS analysis involves staining the cells with fluorescently labeled antibodies that bind to specific surface proteins or markers, which are unique identifiers of particular cell types or states.

[00228] To assess cell phenotype, myeloid progenitor cells were thawed and a cell count was performed. Aliquoted samples of cells were resuspended in surface & later intracellular marker staining cocktails (*e.g.*, fluorescent markers for CD45, CD11b, CD14, CD66b, Ki67, and pHH3). Samples were analyzed on the CytoFLEX LX and data was analyzed using FlowJo software.

[00229] **FIGS. 3A-3D** show exemplary FACS data collected to characterize iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12. **FIGS. 3A and 3B** show genetically engineered cells express well-established myeloid progenitor cell markers, CD45+, CD11b+, and CD14+. **FIG. 3C** shows an exemplary FACS profile that confirm the myeloid progenitor cells do not express neutrophil marker CD66b, supporting the purity of the myeloid progenitor cell population. **FIG. 3D** is an exemplary FACS profile showing the cells do not express proliferation markers Ki67 and pHH3.

[00230] An advantageous aspect of the genetically engineered myeloid progenitor cells described herein is their potential to express M1 markers, which can offer significant benefits when employed for the treatment of cancer, *e.g.*, glioblastoma. By expressing M1 markers,

the myeloid progenitor cells exhibit enhanced anti-tumor effects through the release of pro-inflammatory cytokines and chemokines that activate immune responses against tumor cells. Moreover, the ability of myeloid progenitor cells to penetrate tumor tissues, coupled with their potential to modulate certain immune responses and inhibit tumor-associated angiogenesis, further augments their therapeutic utility. Accordingly, the upregulation of M1 markers from the genetically engineered myeloid progenitor cells was investigated.

[00231] FIG. 4 shows exemplary experimental results confirming iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12 upregulate M1 markers, *i.e.*, HLADR, and CD86. The results are bar graphs based on FACS data collected from myeloid progenitor cell lines that were co-cultured with T cells. These data show that engineered myeloid progenitor cells (CD19t-IL-12, IL-12 monoallelic, and IL-12 biallelic cell lines), but not control cells (non-edited myeloid progenitor cells), upregulated M1 markers when co-cultured in the presence of T cells. The upregulation of the M1 markers highlights the therapeutic efficacy of the iPSC-derived myeloid progenitor cells as the M1 markers are involved in the cells' ability to evoke a potent immune response. For example, the HLADR marker, a major histocompatibility complex class II cell surface receptor, plays a role in presenting antigens to T cells, thereby contributing to the activation of the immune response and anti-tumor activity. Similarly, CD86 is a significant co-stimulatory molecule that enhances T-cell activation, promoting a robust immune reaction.

[00232] FIG. 5 shows exemplary experimental results demonstrating M2 markers (CD206, CD163, and MerTK) were downregulated by the genetically engineered myeloid progenitor cells after co-culture with T cells. The results are bar graphs based on FACS data collected from myeloid progenitor cell populations co-cultured with T cells. These data show that the engineered myeloid progenitor cells (CD19t-IL-12, IL-12 monoallelic, and IL-12 biallelic), but not control cells (non-edited myeloid progenitor cells), downregulated genes that are typically present in tumor-associated macrophages and M2 macrophages, which are known to suppress immune responses and promote tissue remodeling, aiding tumor growth and survival.

[00233] The downregulation of CD206, a mannose receptor associated with alternative macrophage activation (M2-type), signifies a reduced tendency towards a tumor-promoting phenotype. Similarly, the decrease in CD163, another M2 macrophage marker involved in anti-inflammatory responses, reinforces the shift away from a tumor-supportive profile.

Additionally, the reduction in MerTK, a receptor tyrosine kinase that can mediate efferocytosis and contribute to immune evasion by tumors, further underscores the useful characteristics of the genetically modified cells. Hence, the lowered expression of these markers suggests the skewing of genetically engineered myeloid progenitor cells away from an M2 phenotype and towards a more tumoricidal M1 profile.

[00234] Next, the cytokine secretion profiles of iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12 was evaluated. Evaluation of the cytokine secretion profiles provides insight into the functional characteristics of the cell populations – specifically, their ability to produce and release certain cytokines involved in modulating an immune response.

[00235] To evaluate cytokine production, flow-cytometry based approaches were employed. In particular, spent medium was harvested from populations of the genetically engineered myeloid progenitor cells cultured for 5 days and treated with fluorescent markers specific to cytokines of interest, *e.g.*, IL-4, IL-2, IP-10, IL-1 beta, TNF-alpha, MCP-1, IL-17A, IL-6, IL-10, IFN-gamma, IL-12, IL-8, and TGF-beta 1. The presence or absence of the cytokines was then quantified by flow cytometry.

[00236] **FIG. 6** shows exemplary experimental results of cytokine profiles for iPSC derived myeloid progenitor cells genetically engineered to secrete IL-12 and control cells. The results are bar graphs based on flow-cytometry experiments performed to detect cytokines of interest. These data show a side-by-side comparison of cytokine secretion profiles for two genetically engineered myeloid progenitor cell lines (CD19t-IL-12, and IL-12 monoallelic) and control cells (non-edited myeloid progenitor cells). The cytokines assayed included IL-4, IL-2, IP-10, IL-1 beta, TNF-alpha, MCP-1, IL-17A, IL-6, IL-10, IFN-gamma, IL-12, IL-8, and TGF-beta 1.

Production of IL-12 from genetically modified myeloid progenitor cells

[00237] Subsequently, an evaluation of the IL-12 output from the genetically modified myeloid progenitor cells was conducted. To determine the IL-12 production of the genetically engineered myeloid progenitor cells, an assay that made use of Förster Resonance Energy Transfer (FRET) technology was employed. In particular, a homogeneous time resolved fluorescence (HTRF) assay was employed to measure IL-12 production from myeloid

progenitor cells. This sensitive and robust assay was selected for its ability to provide precise quantification of IL-12 production from iPSC-derived myeloid progenitor cell lines. First, populations of myeloid progenitor cells were plated into tissue culture dishes. After 24 hours, spent medium was collected and a cell count was performed. Samples of the spent medium were processed using manufacture's protocols. In particular, donor and acceptor antibodies were incubated in the spent medium for periods ranging from 2 to 24 hours, followed by analysis on a Clariostar microplate reader (BMG Labtech). The readout from this analysis was based on the ratio of acceptor and donor emission signals, providing a measure known as the delta ratio. This parameter, calculated as the difference between the ratio of a standard or sample and the zero-standard ratio, serves as a reliable indicator of IL-12 secretion.

[00238] FIG. 7 provides exemplary experimental results showing IL-12 output of iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12. In particular, these exemplary data show rates of IL-12 production of control cells (non-edited myeloid progenitor cells) and myeloid progenitor cells generated from iPSCs genetically engineered to secrete IL-12 with three different engineering strategies. The rate of IL-12 produced is identified on the y-axis in nanograms per milliliter from cells cultured at a density of one million cells per twenty-four hours (IL-12 ng/mL/1M/24hr). The control and genetically engineered myeloid progenitor cell lines are identified on the x-axis.

[00239] The data obtained from the experiments demonstrate that genetically engineered myeloid progenitor cells, in contrast to control cells, produce a detectable amount of IL-12 for a period of up to 24 hours. These data establish that the genetic modification employed in the engineered cells plays a role in influencing the production of IL-12. The observed differences in IL-12 levels underscore the importance of the engineering strategy employed, suggesting its direct impact on the functional capabilities of the myeloid progenitor cells. These results provide evidence supporting the efficacy and versatility of the disclosed genetic engineering approach for manipulating IL-12 production in myeloid cells.

[00240] Overall, these exemplary results demonstrate the functional attributes of the genetically engineered myeloid progenitor cells, confirming their ability to secrete IL-12 at a level that can contribute to the immunostimulation involved in effective cancer treatment.

Example 4: Genetically engineered myeloid progenitor cells induce immune response and kill tumor cells

[00241] This example describes experimental work that was performed to explore the impact of iPSC-derived myeloid progenitor cells, genetically engineered to secrete IL-12, on tumor cell killing. The genetically engineered myeloid progenitor cells were generated as described in Example 1 and Example 2. As demonstrated below, these experimental results show genetically engineered myeloid progenitor cells, in combination with T cells, trigger an immune response that causes the successful killing of tumor cells. Accordingly, this example provides experimental evidence that genetically engineered myeloid progenitor cells can elicit tumor cell killing, thus providing valuable strategies for therapeutic applications in cancer treatment.

In vitro evaluation of iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12 to induce tumor cell killing

[00242] The methods below were used to evaluate the capacity of iPSC-derived myeloid progenitor cells to elicit tumor cell killing and inhibit tumor cell growth.

[00243] U251NucRed tumor cells were prepared and put into tissue culture plates. Next, engineered myeloid cells and control cells were added at varying densities into the wells. Afterwards, a batch of expanded CD8 T cells sourced from a healthy donor were introduced into the co-cultures, along with suitable stimulation. From Day 0 to Day 5, the co-cultures were evaluated for IL-12 secretion and T cell derived IFN-gamma secretion. At Day 5, the co-cultured cells were stained with the soluble dye Casp-3/7 Green to differentiate cellular viability. Post staining, images of the co-culture were collected and analyzed using the IncuCyte system.

[00244] **FIG. 8** shows exemplary experimental results demonstrating iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12 effectively kill tumor cells and inhibit tumor cell growth in vitro. **FIG. 8** is exemplary data taken from cell killing assays that compare varying quantities of two genetically engineered myeloid progenitor cell lines—one line with CD19t-IL-12 integration, the other with monoallelic, IL-12 integration—and control cells (non-edited myeloid progenitors). The y-axis shows the number of apoptotic U251 cells. The x-axis identifies the cells (and cell populations) present in the corresponding wells. These data establish a trend where an increase in the number of genetically engineered

myeloid progenitor cells secreting IL-12, leads to a rise in the number of apoptotic U251 cells. These results also present the surprising finding that non-engineered, iPSC-derived myeloid progenitor cells (control cells) possess the ability to kill tumor cells despite a reduced capacity to secrete IL-12.

[00245] FIGS. 9A-9B show exemplary experimental results demonstrating the ability of genetically engineered myeloid progenitor cells to kill tumor cells leading to increased tumor cell death. These figures show bar graphs quantifying the growth of U251 tumor cells in co-culture with CD8 T cells and varying quantities of control cells (non-edited myeloid progenitor cells) (FIG. 9A), as compared with genetically engineered myeloid progenitor cell lines having a monoallelic IL-12 (FIG. 9B). The x-axis represents cell numbers for each cell type, while the y-axis corresponds to U251 tumor cell growth. Measurements were taken after a 5-day co-culture period. Notably, the data reveals that IL-12 engineered myeloid progenitor cells effectively inhibited cancer growth over a range of cells/well, while the non-edited myeloid progenitor cells (parental cell line) had negligible impact on cancer growth. Together these data demonstrate IL-12 engineering enhances myeloid progenitor mediated tumor killing by T cells.

Example 5: Genetically engineered myeloid progenitor cells demonstrate resistance to the tumor microenvironment and drive an enhanced immune response

[00246] This example describes experimental results that was collected to evaluate the impact of the tumor microenvironment on the ability of engineered myeloid progenitor cells to elicit a pro-inflammatory, anti-tumor response from CD8 T cells. The exemplary results described below provide evidence as to the capabilities of iPSC-derived myeloid progenitor cells to overcome an immunosuppressive tumor microenvironment (evaluated by M2 polarization) and elicit CD8 T cell activity, an important component of an anti-tumor immune response.

[00247] FIG.10 illustrates an exemplary workflow that was used to evaluate the impact of M2 polarization on CD8 T cell activity mediated by genetically modified myeloid progenitor cells. Myeloid progenitor cells derived from iPSCs genetically engineered to secrete IL-12 and control cells (myeloid progenitor cells derived from non-edited iPSCs) were cultured, separately, under conditions that simulate the tumor microenvironment. This stimulation involved culturing cell populations in the presence of TGF-beta and IL-10 to induce M2

skewing. In parallel, CD8 T cells were separated from donor PBMCs using a commercially available CD8 T cell isolation kit. Subsequently, the isolated CD8 T cells were co-cultured with M2-skewed myeloid progenitor cells. Following this co-culture period, the tissue culture dishes containing populations of modified progenitor cells were evaluated to measure the levels IL-12, IFN-gamma, TNF-alpha, IL-2, Perforin, Granzyme A, Granzyme B, and Granulysin in the cell supernatant. Exemplary experimental results of this workflow are reported in **FIGS. 11A, 11B**, and **FIGS. 12A-12G**.

[00248] **FIGS. 11A and 11B** are exemplary experimental results that show myeloid progenitor cells rescue IFN-gamma secretion from CD 8 T cells despite M2 polarization. Specifically, **FIG. 11A** shows the amounts of IFN-gamma (y-axis) that was detected by flow cytometry following the co-culture of T cells with varying quantities of M2 polarized myeloid progenitor cells (indicated on x-axis). This data demonstrate that the secretion of IFN-gamma by T cells is rapidly elevated by the addition of genetically engineered myeloid progenitor cells despite M2 polarization. The M2 polarized myeloid engineered progenitor cells were derived from iPSCs genetically engineered to secrete IL-12 as described herein. **FIG. 11B** is exemplary data showing the amounts of IL-12 (y axis) that was detected by varying quantities of M2 polarized myeloid progenitor cells (identified along x-axis). Taken together, these data demonstrate that myeloid progenitor cells derived from iPSCs genetically engineered to secrete IL-12 can stimulate activation of CD8 T cell anti-tumor function despite M2 skewing.

[00249] **FIGS. 12A-12G** are exemplary experimental results showing myeloid progenitor cells genetically engineered to secrete IL-12 activate CD8 T cell cytolytic activity despite M2 polarization. More particularly, **FIG. 12A** shows the amount of IL-12 (indicated on y-axis) that was detected from the cell populations indicated on the x-axis. **FIGS. 12B-12G** show the amounts of CD8 T cell cytolytic cytokines (TNF-alpha, IL-2, Perforin, Granzyme A, Granzyme B, and Granulysin - as indicated) in that was detected from corresponding cell populations.

[00250] Taken together, these exemplary findings underscore the capabilities of genetically engineered myeloid progenitor cells to overcome the immunosuppressive effects of the tumor microenvironment, as demonstrated by M2 polarization, to stimulate broad pro-inflammatory CD8 T cell activity including enhanced pro-inflammatory cytokine production and increased cytolytic effector functions.

Example 6: Myeloid progenitor cells genetically engineered to secrete IL-12 demonstrate significant tumor suppression *in vivo*

[00251] This example describes experimental work that was performed to evaluate the therapeutic capacity of genetically engineered myeloid progenitor cells to treat a tumor, *in vivo*. This example also provides exemplary experimental data that present compelling evidence of significant tumor suppression that was achieved with myeloid progenitor cells genetically engineered to secrete IL-12 as described herein.

[00252] To assess the anti-tumor effectiveness of myeloid progenitor cells *in vivo*, a humanized mouse model of glioblastoma (GBM) was used. The study involved implanting GBM tumor cells into the flank of immune compromised mice. Following this, human peripheral blood mononuclear cells (huPBMCs) were adoptively transferred into the mice. Intratumoral (IT) injections of both control cells and genetically engineered myeloid progenitor cells were administered directly into the flank tumors. Over the course of the experiment, tumor growth was closely monitored, and the anti-tumor efficacy of the myeloid progenitor cells was evaluated over time.

[00253] **FIG. 13** provides exemplary data showing the significant tumor suppressive capabilities of genetically engineered myeloid progenitor cells, *in vivo*. Specifically, **FIG. 13** shows line graphs of tumor volumes (indicated on y-axis) that were detected over time (in days, indicated on x-axis) from mice across different treatment groups. The upper panel shows all mice in the study and the lower panels shows individual mice per treatment group. These exemplary data demonstrate the anti-tumor efficacy of genetically engineered myeloid progenitor cells. Notably, the data indicate that myeloid progenitor cell lines engineered to express IL-12 (IL-12 CD19t and IL-12 biallelic) exhibited superior tumor suppressive capabilities when compared to no treatment or control myeloid progenitor cell treatment groups.

[00254] **FIGS. 14A and 14B** show exemplary experimental results further demonstrating the *in vivo* therapeutic efficacy of genetically engineered myeloid progenitor cells. **FIG. 14A** shows exemplary data that was obtained by collecting tumor measurements from pre-clinical mouse models of glioblastoma (GBM) that had been subjected to the indicated treatments (shown on the x-axis). These exemplary data clearly demonstrate a significantly lower tumor growth inhibition ratio (y-axis) when using myeloid progenitor cells genetically engineered to

secrete IL-12 compared to treatment with control non-edited myeloid progenitor cells. Importantly, the anti-tumor efficacy of *in vivo* treatment with genetically engineered myeloid progenitor cells was seen with multiple engineering strategies to increase IL-12 production in the TME. Surprisingly, these exemplary data show administration of genetically engineered myeloid progenitors to the TME results in greater tumor growth inhibition than aPD1 treatment, highlighting the therapeutic capacity of the approach described herein for treatment of solid tumors, including GBM.

[00255] FIG 14B shows representative images of the tumors which were harvested from the pre-clinical mouse model described in FIG 14A at humane end points. The tumors were cleared of surrounding fat and muscle tissue to ensure accurate size measurements. The images demonstrate the animals treated with genetically engineered myeloid progenitor cells exhibited the smallest tumor sizes among all the treatment groups. Notably, the data indicate that myeloid progenitor cell lines engineered to express IL-12 (CD19t-IL-12 and IL-12 monoallelic) exhibited superior tumor suppressive capabilities when compared to aPD1 monoclonal antibody (mAB) treatment. Furthermore, intratumoral (IT) treatment with genetically engineered myeloid progenitor cells led to a greater reduction in tumor growth compared to aPD1 treatment. This provides visual evidence that further supports the exceptional tumor-suppressive capabilities of the genetically engineered myeloid progenitor cells in comparison to the other treatment approaches.

[00256] Next, the temporal dynamics of IL-12 expression and safety profile based on serum cytokine levels were evaluated in pre-clinical GBM models as described herein. Serum IL-12 levels were evaluated over time in mice treated with genetically engineered myeloid progenitor cells, control cells, and a PD1 treatment.

[00257] FIG. 15 shows IL-12 levels detected in the serum of mice with flank GBM tumors over a 20-day period. Serum samples were collected from the animals at specific time points as indicated, and IL-12 secretion was analyzed using bead-based Luminex assay. The data demonstrate that animals treated with genetically engineered myeloid cells exhibited detectable levels of IL-12 in their serum. These findings indicate the sustained and durable expression of IL-12 for a minimum of 20 days following the administration of the myeloid progenitor cells to the tumor site. These data highlight the extended presence of IL-12, which holds significant implications for the therapeutic capacity and longevity of the treatment approach utilizing genetically engineered myeloid progenitor cells. Furthermore, these data

demonstrate that IT treatment with genetically engineered myeloid progenitor cells results in more IL-12 production than systemic aPD1 mAb treatment as reported herein.

[00258] FIGS 16A-16C provide exemplary results showing serum cytokine levels associated with cytokine release syndrome (CRS), in mice treated with genetically engineered myeloid progenitor cells or control cells as compared to treatment with anti-human PD-1 antibody (aPD1). The evaluation was performed on the IT treatment with genetically engineered myeloid progenitor cells (CD19t-IL-12) in comparison to the benchmark monoclonal antibody (mAb) treatment aPD1 administered intraperitoneally (IP). The objective was to compare the levels of cytokines in the serum known to drive CRS following the respective treatments. The data were obtained from humanized mice, where human flank GBM tumors were established, followed by the transfer of human peripheral blood mononuclear cells (huPBMCs). Subsequently, the mice received either the myeloid progenitor cell treatment or aPD1 treatment. The evaluation of CRS was performed by analyzing the serum cytokine levels via Luminex at day 14. **FIG. 16A** shows levels of IL-6 (pg/ml) on the Y axis compared across treatment groups: untreated, vehicle, control cells, genetically engineered myeloid progenitor cells (CD19t-IL-12), and aPD1. **FIG. 16B** shows levels of IL-8 (pg/ml) on the Y axis compared across treatment groups: untreated, vehicle, control cells, genetically engineered myeloid progenitor cells (CD19t-IL-12), and aPD1. **FIG. 16C** shows levels of IL1-beta (pg/ml) on the Y axis compared across treatment groups: untreated, vehicle, control cells, genetically engineered myeloid progenitor cells (CD19t-IL-12), and aPD1.

[00259] The results demonstrate that treatment with genetically engineered myeloid progenitor cells, as provided herein, exhibits a safety profile comparable to the standard aPD1 treatment given widely to patients with solid tumors. The analysis of serum cytokines known in the field to be players in CRS further supports the favorable safety profile of the myeloid progenitor cell treatment. These findings highlight the ability of iPSC-derived myeloid progenitor cells genetically engineered to secrete IL-12, as disclosed here, to be used as a safe and alternative therapy to the antibody-based therapies now available to patients with solid tumors.

Example 7: Genetically engineered myeloid progenitor cells reshape the tumor microenvironment

[00260] This example describes experimental work that was conducted to evaluate the capacity of genetically engineered myeloid progenitor cells to re-polarize the tumor microenvironment (TME). In particular, this example describes experimental results that shed light onto the effects of administering genetically engineered myeloid progenitor cells on the TME. The results obtained from our experimental analyses provide evidence of a notable phenomenon: the administration of genetically engineered myeloid progenitor cells led to a significant increase in the infiltration of CD3 and CD8 T cells into the TME. This observation highlights the ability of genetically engineered myeloid progenitor cells to reconfigure the immunological landscape of the TME, fostering an environment conducive to enhanced anti-tumor immune cell presence and activity. These findings demonstrate the use of myeloid progenitor cells as a therapeutic intervention to modulate and remodel the tumor microenvironment, thereby facilitating a more robust and effective anti-tumor T cell mediated immune response.

[00261] FIG. 17A-17D show exemplary experimental results that demonstrate the remodeling of the tumor microenvironment (TME) towards an anti-tumor cytolytic T lymphocyte (CTL) response following the administration of myeloid progenitor cells. In FIG. 17A, experiment data showing the ratios of anti-tumor cytolytic CD8 T cells to pro-tumor immunosuppressive FoxP3⁺ T cells (Tregs) in the tumor after treatment with myeloid progenitor cells genetically engineered to secrete IL-12 or control cells (non-edited myeloid progenitor cells). These data provide evidence that treatment with genetically engineered myeloid progenitor cells leads to an increase in the CD8 to Treg ratios in the TME, indicative of a repolarization of the TME towards an anti-tumor CTL response.

[00262] Further evidence of the TME repolarization is provided by FIGS. 17B-17D. For example, FIG. 17B demonstrates an infiltration of T cells (CD3⁺) within the TME with an overall decreased exhaustion phenotype (PD1 negative and TIM3 negative), indicating a shift from immunosuppressive to pro-inflammatory phenotypes, supporting the notion of repolarization. Additionally, FIG. 17B shows an increased frequency of total T cells within the TME, and FIGS. 17C and 17D show a decrease in exhaustion markers (PD1/TIM3) within CD4 and CD8 subsets in the TME. This observation suggests that the shift in T cell frequency is accompanied by a functional change in T cell activation in the TME by *in vivo* intratumoral (IT) treatment with genetically engineered myeloid progenitor cells. Together, these data show *in vivo* treatment with genetically edited myeloid progenitor cells leads to a

decreased exhaustion phenotype (Tim3 and PD1) in conjunction with an increased CD8 to Treg ratio in the TME, indicating the favorable repolarization of the TME to facilitate increases in anti-tumor T cell activation and decreases in immunosuppressive and exhausted T lymphocyte populations.

[00263] Together, these exemplary experimental results provide evidence of the remodeling of the tumor microenvironment towards a pro-tumor cytolytic T lymphocyte response following the administration of genetically engineered myeloid progenitor cells. These findings underscore the value of these cells as a therapeutic strategy for promoting a robust and effective anti-tumor immune response by repolarizing the immunosuppressive TME towards an anti-tumor pro-inflammatory and cytolytic state.

Example 8: Enhanced anti-tumor responses from myeloid progenitor cells derived from iPSCs *ex-vivo*

[00264] The following experiments were conducted to assess the capacity of myeloid progenitor cells to enhance anti-tumor responses. These experiments demonstrate that myeloid progenitors, generated *ex vivo* from iPSCs, retain a remarkable ability to rescue CD8 anti-tumor cytokines production even despite M2 polarization. These data point to the therapeutic efficacy of myeloid progenitor cells even in the context of a tumor microenvironment.

[00265] Briefly, myeloid progenitor cells were generated from iPSCs, as described in Example 1, and polarized to an M2 phenotype by the addition of TGF β and IL-10 to cell cultures. The myeloid progenitor cells were then cultured with CD8 T cells under conditions in which an anti-CD-28 monoclonal antibody (mAb) was either present or absent from the cultures.

[00266] **FIGS. 18A and 18B** demonstrate that the myeloid progenitor cells rescue CD8 T cell effector cytokines without agonistic CD28 mAb. In particular, these data show that engineered M2 polarized, myeloid progenitor cells can restore both CD8 IFN γ (**FIG. 18A**) and TNF α (**FIG. 18B**) to a level of pro-inflammatory cytokine rescue that is comparable to CD8 T cells stimulated with or without anti-CD28 mAb. **FIG. 18A** shows that levels of IFN γ secreted from CD8 T cells is comparable to when the CD8 T cells are cultured with M2 myeloid progenitor cells with or without anti-CD28 mAb relative to CD8 T cells only or

CD8 T cells with anti-CD3 mAb alone and without anti-CD28 mAb. **FIG. 18B** shows that levels of TNF- α secreted from CD8 T cells is comparable to when the T cells are cultured with M2 myeloid progenitor cells with and without anti-CD28 mAb relative to CD8 T cells only or CD8 T cells with anti-CD3 and without anti-CD28 mAb controls. Taken together these data show that myeloid progenitor cells can enhance CD8 anti-tumor cytokine production even in the presence of an M2 phenotype, demonstrating their usefulness in tumor therapy and the impact of the intrinsic myeloid progenitor cell, which provides co-stimulation through CD28 activation, resulting in comparable levels of T cell activation to exogenous anti-CD28 mAb.

[00267] Experiments were also conducted to assess whether iPSC-derived myeloid progenitor cells can induce CD8 tumor killing without agonistic CD28 mAb. Briefly, CD8 T cells were cultured with U251 tumor spheroids in a monolayer with the myeloid progenitor cells or control cells under the stimuli indicated.

[00268] Exemplary results are shown in **FIGS. 19A and 19B**. Myeloid progenitor cells were able to induce CD8 tumor cell killing in the presence of anti-CD3 alone (**FIG. 19A**). Treatment with anti-CD3 and anti-CD28 (**FIG. 19B**) resulted in comparable levels of CD8 tumor cell killing by myeloid progenitor cells as treatment with anti-CD3 alone. These data, together with the observation that myeloid progenitor cells increase CD28 ligands CD80/CD86 on its surface, suggest that myeloid progenitor cells, generated *ex vivo* from iPSCs as described in Example 1, provide CD28 engagement on T cells, through CD80/86, to drive CD8 tumor killing activity. These data also demonstrate the therapeutic efficacy of the myeloid progenitor cells on tumor size and highlight the ability of the myeloid progenitor cell to activate anti-tumor effector responses.

[00269] Experiments were also conducted to assess whether iPSC-derived myeloid progenitor cells can promote pro-inflammatory cytokine (IFN γ and TNF α) production from Tregs. Briefly, varying amounts of the myeloid progenitor cells (either 17,000, 35,000, or 70,000) were co-cultured with or without a set number of Tregs.

[00270] Exemplary results are shown in **FIGS. 20A and 20B**. These results demonstrate myeloid progenitor cells, derived *ex-vivo* from iPSCs as described in Example 1, promote pro-inflammatory cytokine (IFN γ and TNF α) production from Tregs. Co-culturing of Tregs

with myeloid progenitor cell stimulated higher production of IFN γ and TNF α from Tregs than when the Tregs were cultured alone.

EQUIVALENTS AND SCOPE, INCORPORATION BY REFERENCE

[00271] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above description, but rather is as set forth in the appended claims.

[00272] In the claims articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or all of the group members, are present in, employed in, or otherwise relevant to a given product or process.

[00273] Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the claims or from relevant portions of the description is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

[00274] Where elements are presented as lists, *e.g.*, in Markush group format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be

removed from the group. It is also noted that the term “comprising” is intended to be open and permits the inclusion of additional elements or steps. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, steps, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, steps, etc. Thus, for each embodiment of the invention that comprises one or more elements, features, steps, etc., the invention also provides embodiments that consist or consist essentially of those elements, features, steps, etc.

[00275] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. It is also to be understood that unless otherwise indicated or otherwise evident from the context and/or the understanding of one of ordinary skill in the art, values expressed as ranges can assume any subrange within the given range, wherein the endpoints of the subrange are expressed to the same degree of accuracy as the tenth of the unit of the lower limit of the range.

[00276] In addition, it is to be understood that any particular embodiment of the present invention may be explicitly excluded from any one or more of the claims. Where ranges are given, any value within the range may explicitly be excluded from any one or more of the claims. Any embodiment, element, feature, application, or aspect of the compositions and/or methods of the invention, can be excluded from any one or more claims. For purposes of brevity, all of the embodiments in which one or more elements, features, purposes, or aspects is excluded are not set forth explicitly herein.

[00277] All publications, patents, and sequence database entries mentioned herein, including those items listed above, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

CLAIMS

What is claimed is:

1. A population of engineered immune cells that secrete interleukin-12 (IL-12) at a concentration of between 1.0 and 1,000 ng/ml, as measured by homogeneous time resolved fluorescence (HTRF), when said cells are cultured at a density of 1 million cells per milliliter for 24 hours.
2. The population of engineered immune cells of claim 1, wherein the population of immune cells secretes IL-12 at a concentration below 500 ng/ml, as measured by HTRF, when said cells are cultured at a density of 1 million cells per milliliter for 24 hours.
3. The population of engineered immune cells of claim 1 or claim 2, wherein the population of engineered immune cells are genetically modified to comprise a heterologous nucleic acid encoding IL-12.
4. The population of engineered immune cells of claim 3, wherein expression of the heterologous nucleic acid is driven by a promoter sequence of an endogenous gene.
5. The population of engineered immune cells of claim 4, wherein the endogenous gene comprises a sustained transgene expression locus (STEL).
6. The population of engineered immune cells of claim 4, wherein the endogenous gene comprises Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
7. The population of engineered immune cells of any of claims 1-6, wherein the population of immune cells comprises one or more of hematopoietic stem cells, myeloid progenitor cells, microglia precursor cells, microglial cells, dendritic cells, T cells, B cells, natural killer cells, or macrophages.
8. The population of engineered immune cells of claim 7, wherein the population of immune cells is a population of myeloid progenitor cells.

9. The population of engineered immune cells of claim 8, wherein the myeloid progenitor cells are derived from iPSCs.
10. The population of engineered immune cells of any one of claims 1-9, wherein the population of engineered immune cells express CD11b, CD14, and CD45.
11. The population of engineered immune cells of claim 10, wherein at least 80 percent of the engineered immune cells express CD11b, CD14, and CD45.
12. The population of engineered immune cells of any one of claims 1-11, wherein the population of engineered immune cells express HLADR, CD86, CD80, and CD40.
13. The population of engineered immune cells of any one of claims 1-12, wherein the population of engineered immune cells do not express CD206, CD163, or MerTK.
14. The population of engineered immune cells of any one of claims 1-13, wherein the population of immune cells are able to present tumor antigens to other immune cells capable of recognizing antigens.
15. The population of engineered immune cells of claim 14, wherein each cell within the population of the immune cells comprises a major histocompatibility complex (MHC) molecule on the cell surface, and wherein said MHC molecule binds to and presents the tumor antigens to the other immune cells.
16. The population of engineered immune cells of claim 15, wherein said MHC molecule comprises a MHC class I molecule or an MHC class II molecule.
17. The population of engineered immune cells of any of claims 1-16, wherein each cell of the population of immune cells comprises a co-stimulatory molecule on the cell surface, and wherein said co-stimulatory molecule enhances an immune response by interacting with immune cells.

18. The population of engineered immune cells of claim 17, wherein the immune response is a CD8⁺ T cell-mediated immune response.
19. The population of engineered immune cells of claim 17 or claim 18, wherein said co-stimulatory molecule comprises CD80, CD86, or CD40.
20. The population of engineered immune cells of any one of claims 15-19, wherein each of the immune cells comprises one or more genetic alterations that results in increased expression of MHC molecule on the cell surface, thereby increasing the capability of the immune cells to present the tumor antigens to T cells.
21. The population of engineered immune cells of any of claims 1-20, wherein each of the immune cells comprises a targeting moiety on the cell surface.
22. The population of engineered immune cells of claim 21, wherein the targeting moiety comprises an exogenously expressed chimeric or naturally occurring protein capable of binding to an antigen to elicit activation of an immune cell.
23. A method of preparing the population of engineered immune cells of any one of claims 1-22, the method comprising
introducing a heterologous nucleic acid encoding IL-12 into a stem cell using a CRISPR/Cas system; and
deriving the immune cells from the stem cell, thereby producing the population of engineered immune cells.
24. The method of claim 23, wherein the stem cell comprises an induced pluripotent stem cell.
25. The method of claim 24, wherein the heterologous nucleic acid encoding IL-12 is integrated into a sustained transgene expression locus (STEL).
26. The method of claim 25, wherein the STEL loci comprises a GAPDH gene.

27. A pharmaceutical composition for treating a tumor, the composition comprising:
the population of immune cells of any one of claims 1-22; and
a pharmaceutical acceptable carrier, carrier, or dilutant.
28. A method of stimulating an immune response against a tumor, the method comprising:
administering the population of engineered immune cells of any one of claims 1-22;
and
allowing the population of engineered immune cells to secrete IL-12, thereby
stimulating a CD8⁺ T cell-mediated response against the tumor.
29. The method of claim 28, wherein the tumor is a glioblastoma.
30. The method of claim 28 or 29, wherein the administering comprises direct injection of
the population of cells into the site of the tumor.
31. The method of any one of claims 28-30, wherein the population of cells stimulates an
immune response against the tumor in the subject.
32. The method of claim 31, wherein the immune response is a CD8⁺ T cell-mediated
response.
33. The method of claim 31 or 32, wherein the immune response comprises secretion of
pro-inflammatory cytokines by T regs.
34. The method of claim 33, wherein the pro-inflammatory cytokines comprise IFN γ ,
TNF α , or both IFN γ and TNF α .
35. The method of any one of claims 28-34, wherein the method further comprises
administering an additional anti-cancer therapy to the subject.

36. The method of claim 35, wherein the additional anti-cancer therapy comprises a CD28 agonist.
37. The method of claim 35, wherein the additional anti-cancer therapy does not comprise a CD28 agonist.
38. The method of claim 36 or 37, wherein the CD28 agonist is an anti-CD28 antibody.
39. The method of any one of claims 28-38, wherein the subject is a human.
40. A kit comprising a dosage form suitable for administration to a subject comprising the engineered immune cells of any one of claims 1-22, and instructional material for the use of said dosage form.
41. A kit comprising a dosage form suitable for administration to a subject comprising the pharmaceutical composition of claim 19, and instructional material for the use of said dosage form.
42. A device for administering a therapy to a subject comprising the composition of claim 27.
43. An engineered cell comprising a heterologous nucleic acid encoding a cytokine, or a fragment thereof, wherein the heterologous nucleic acid is integrated at or near an endogenous gene.
44. The engineered cell of claim 43, wherein the endogenous gene comprises a sustained transgene expression locus (STEL).
45. The engineered cell of claim 44, wherein the endogenous gene comprises GAPDH.
46. The engineered cell of any of claims 43-46, wherein the cytokine or fragment thereof comprises IL-12.

47. The engineered cell of any of claims 43-46, wherein the engineered cell comprises a stem cell or an immune cell.
48. The engineered cell of claim 47, the immune cell comprises a myeloid progenitor cell.
49. A population of cells derived from the engineered cell of any of claims 43-48, wherein the population of cells express the cytokine at a concentration of between 1.0 and 1,000 ng/ml, as measured by homogeneous time resolved fluorescence (HTRF), when said cells are cultured at a density of 1 million cells per milliliter for 24 hours.
50. The population cells of claim 49, wherein the population of cells secrete the cytokine at a concentration below 500 ng/ml, as measured by HTRF, when said cells are cultured at a density of 1 million cells per milliliter for 24 hours.

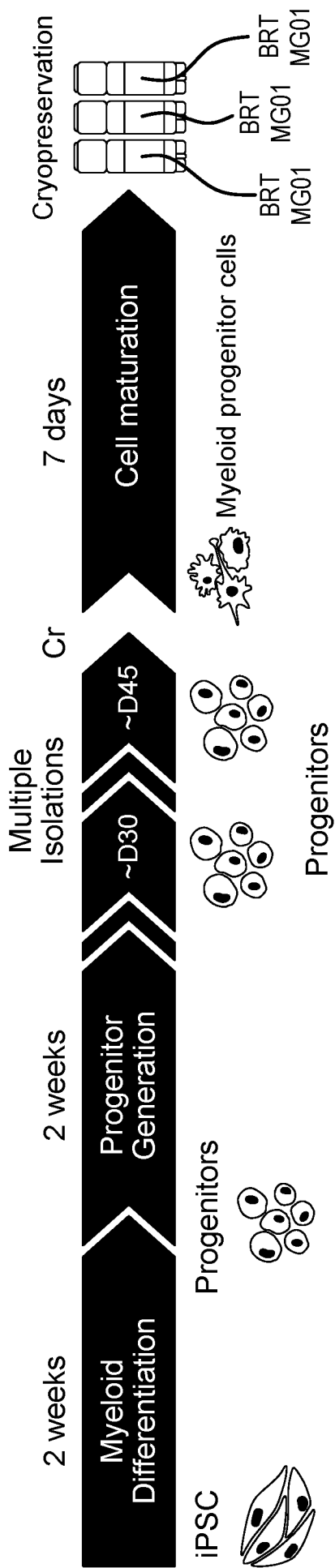


FIG. 1

1/24

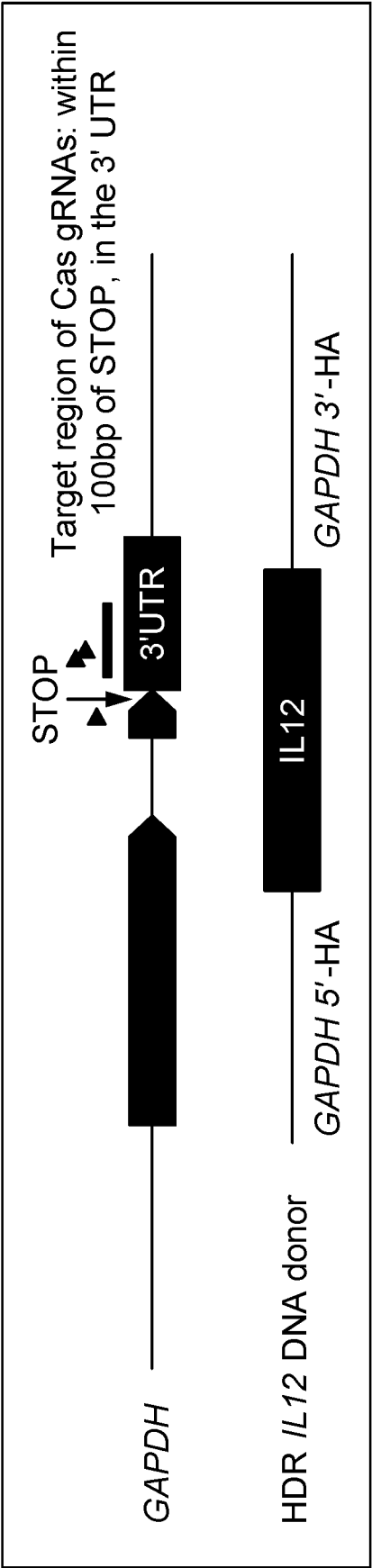


FIG. 2

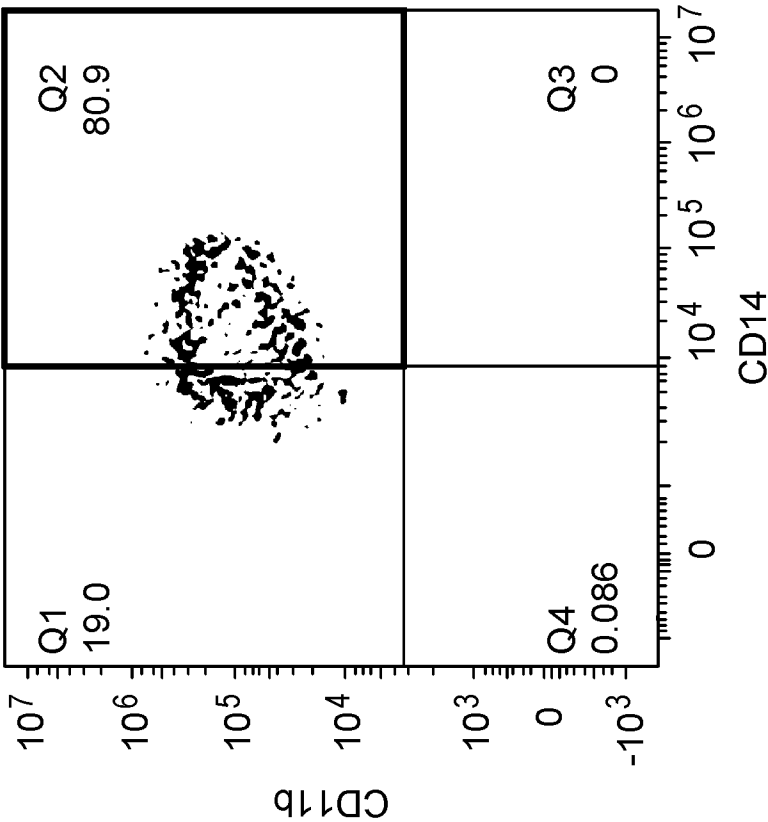


FIG. 3B

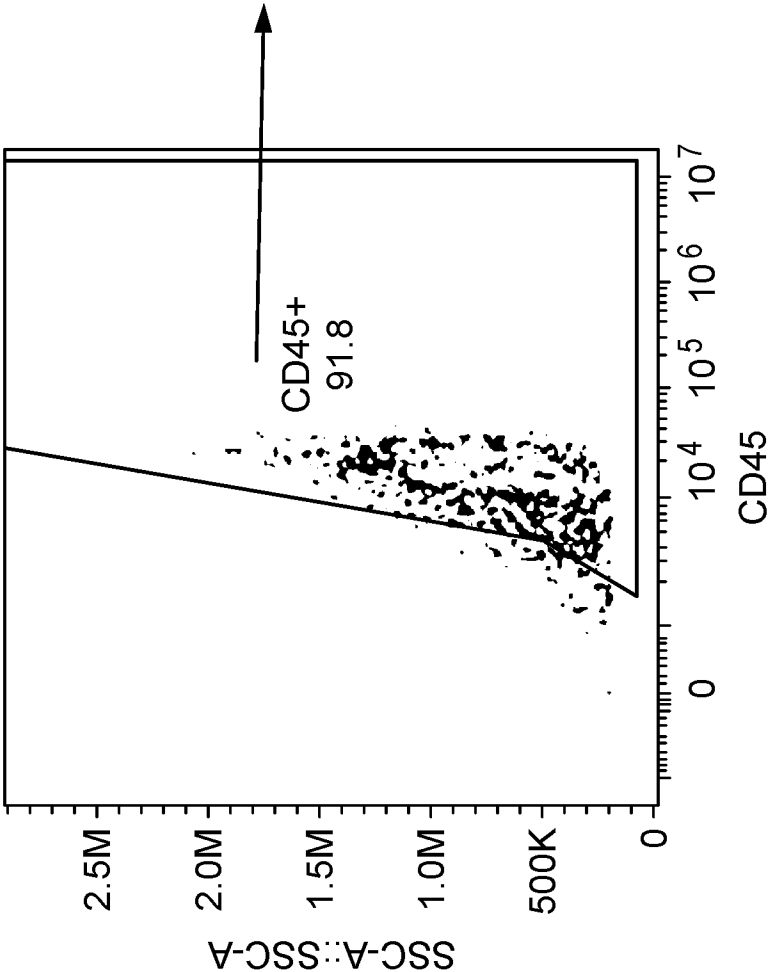


FIG. 3A

3/24

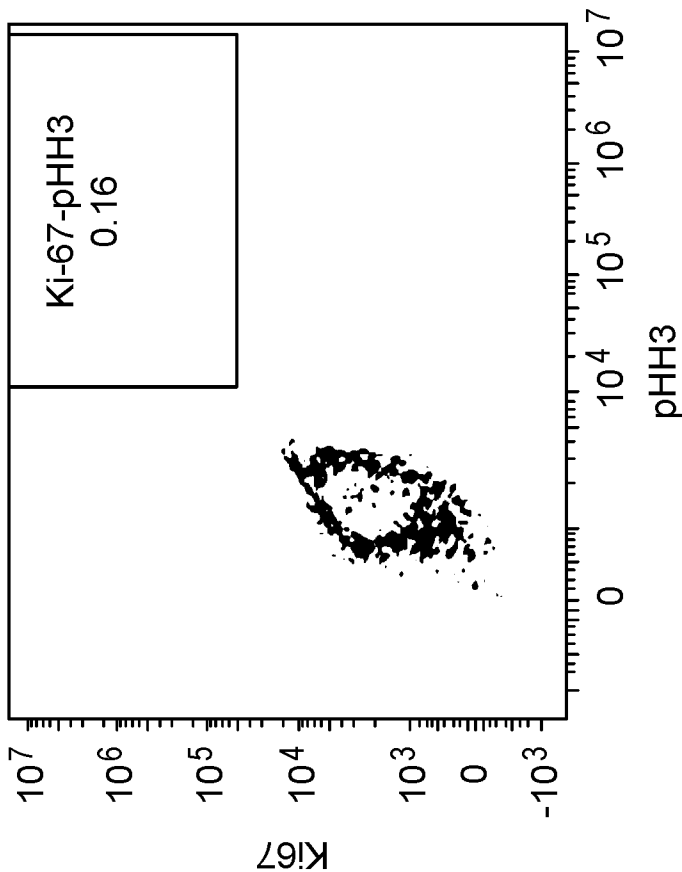


FIG. 3D

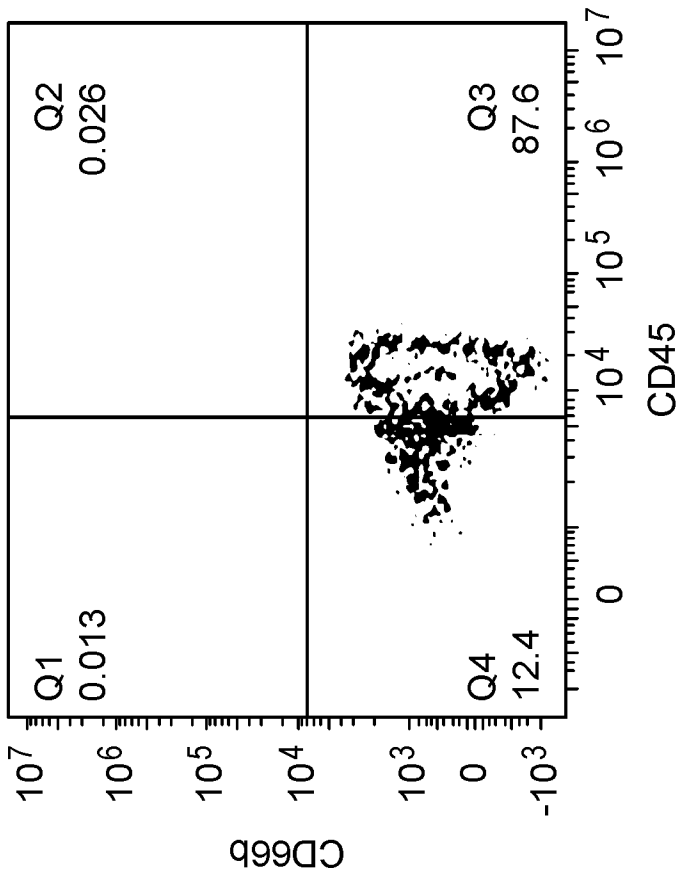
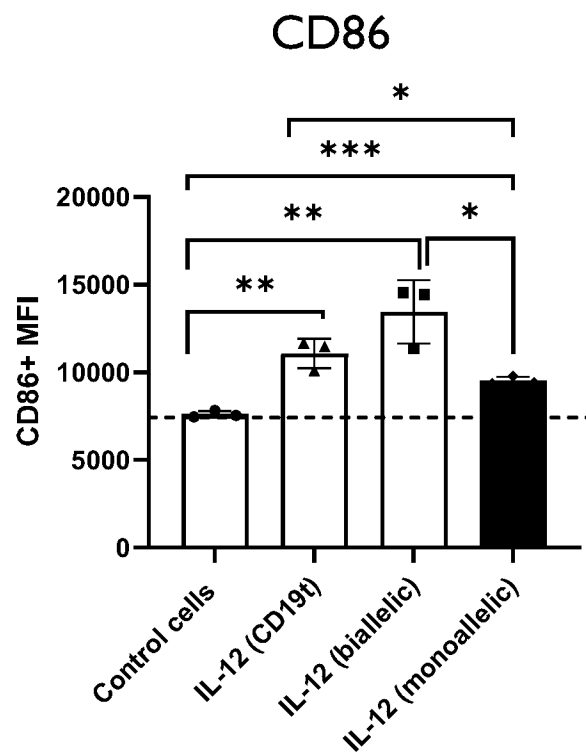
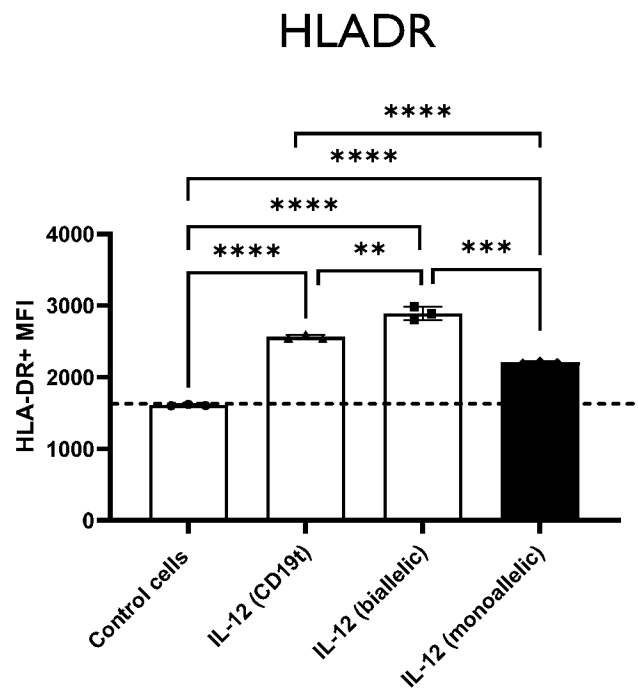
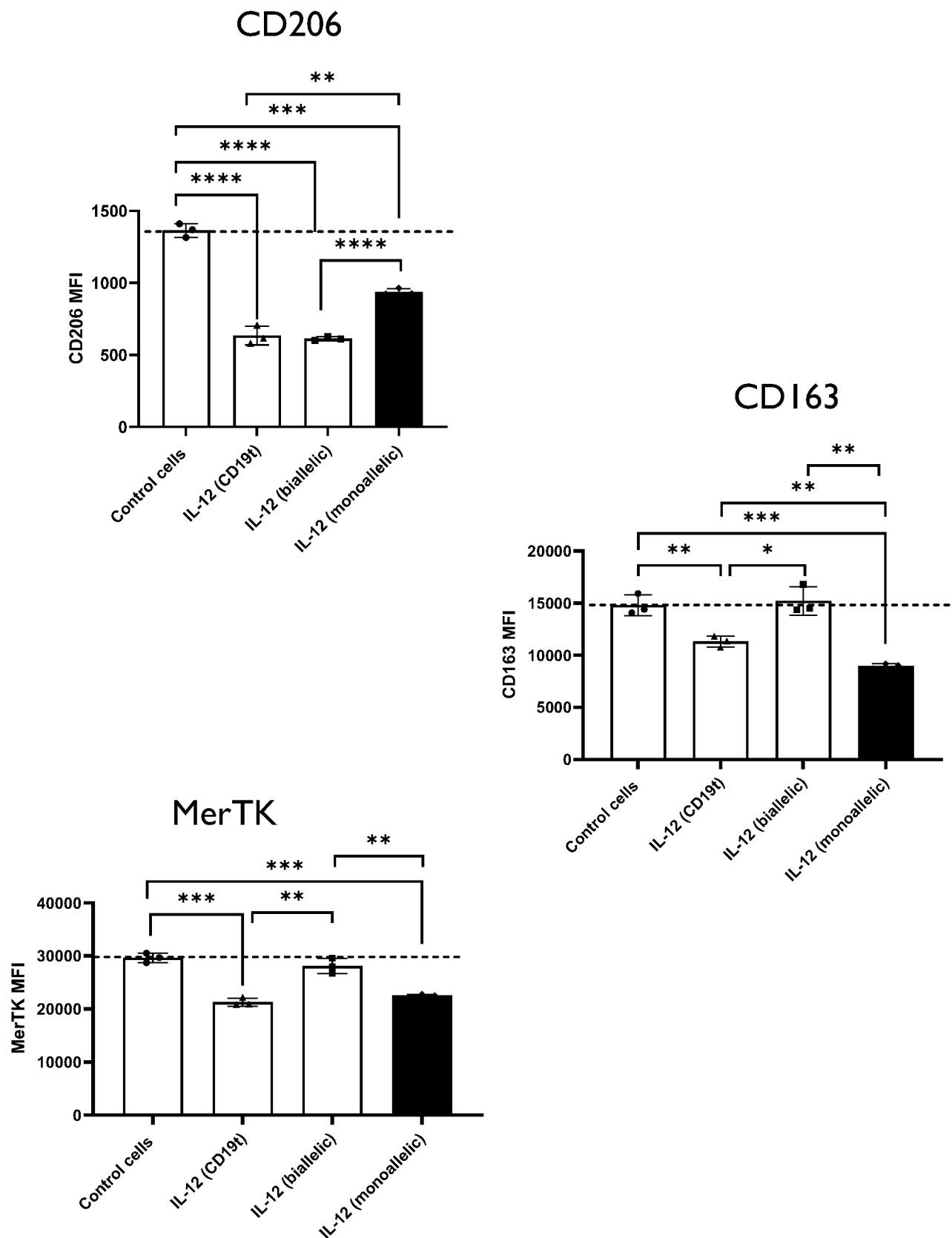


FIG. 3C

4/24

**FIG. 4**

5/24

**FIG. 5**

6/24

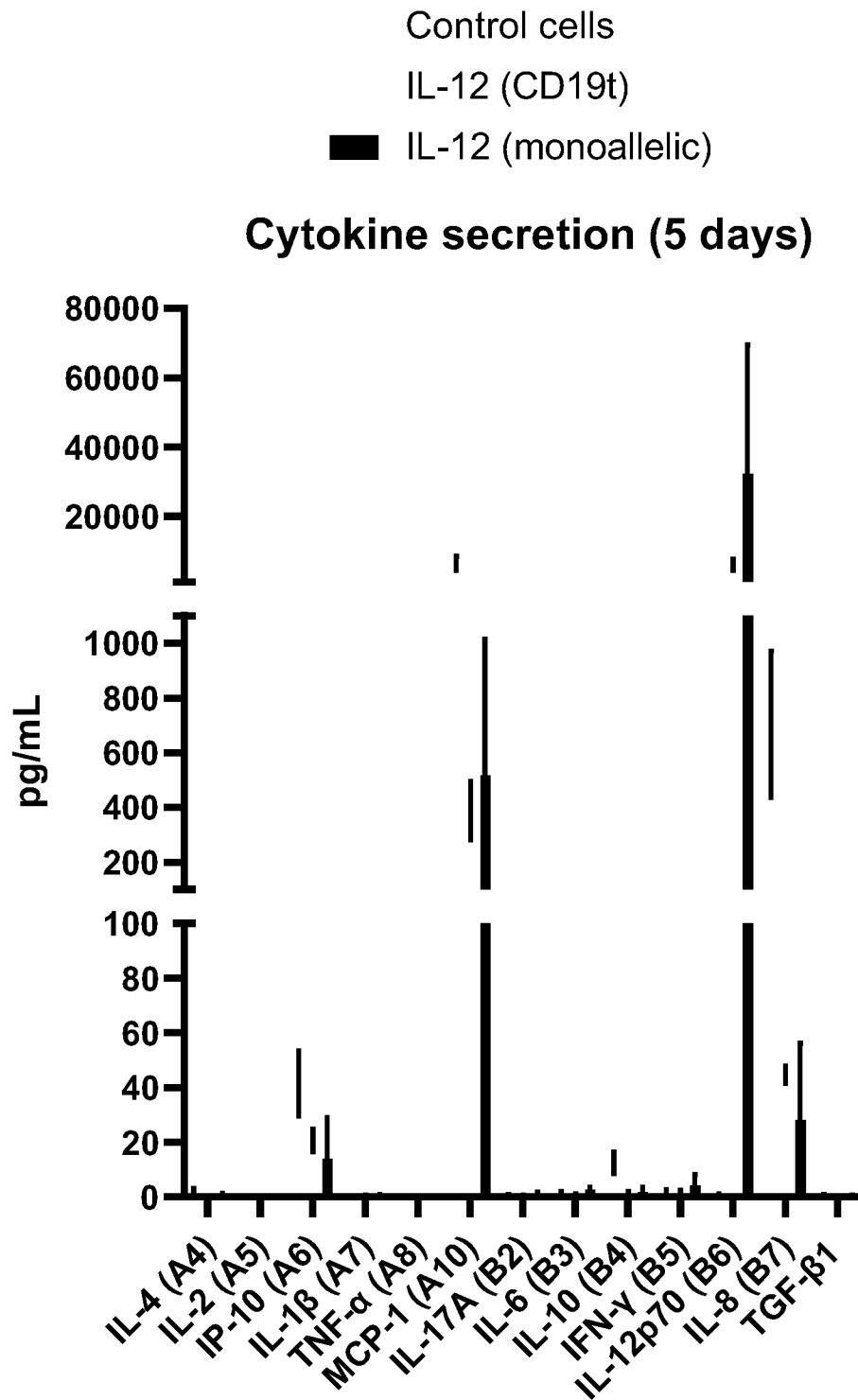


FIG. 6

7/24

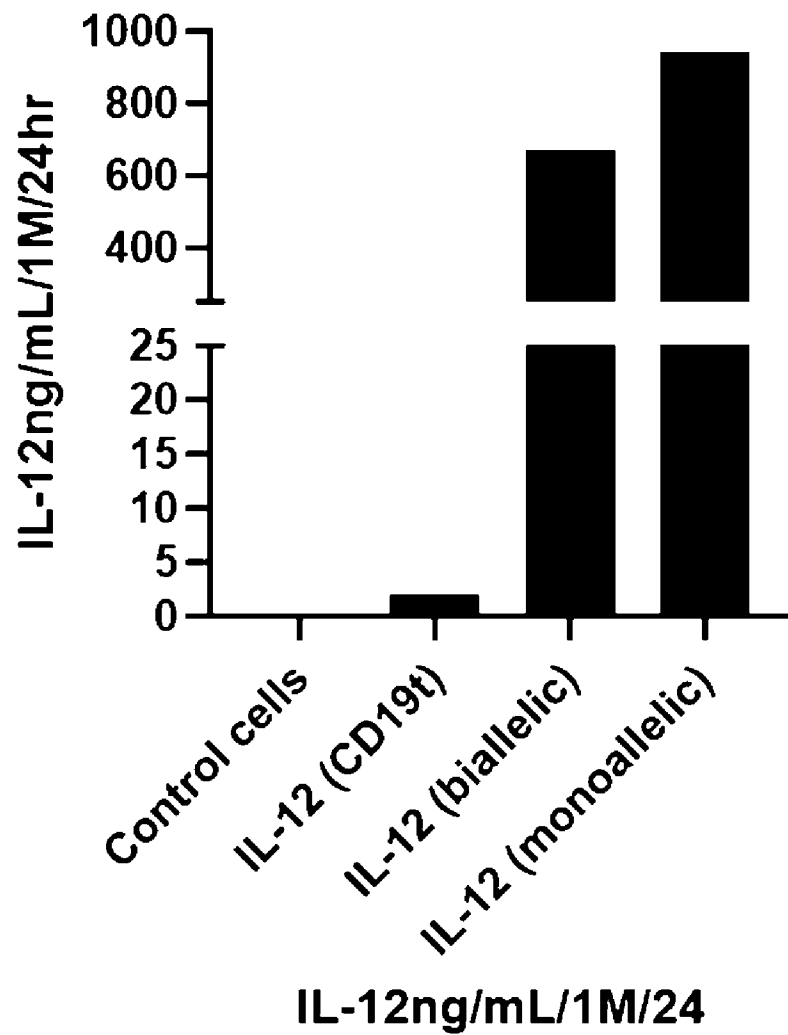
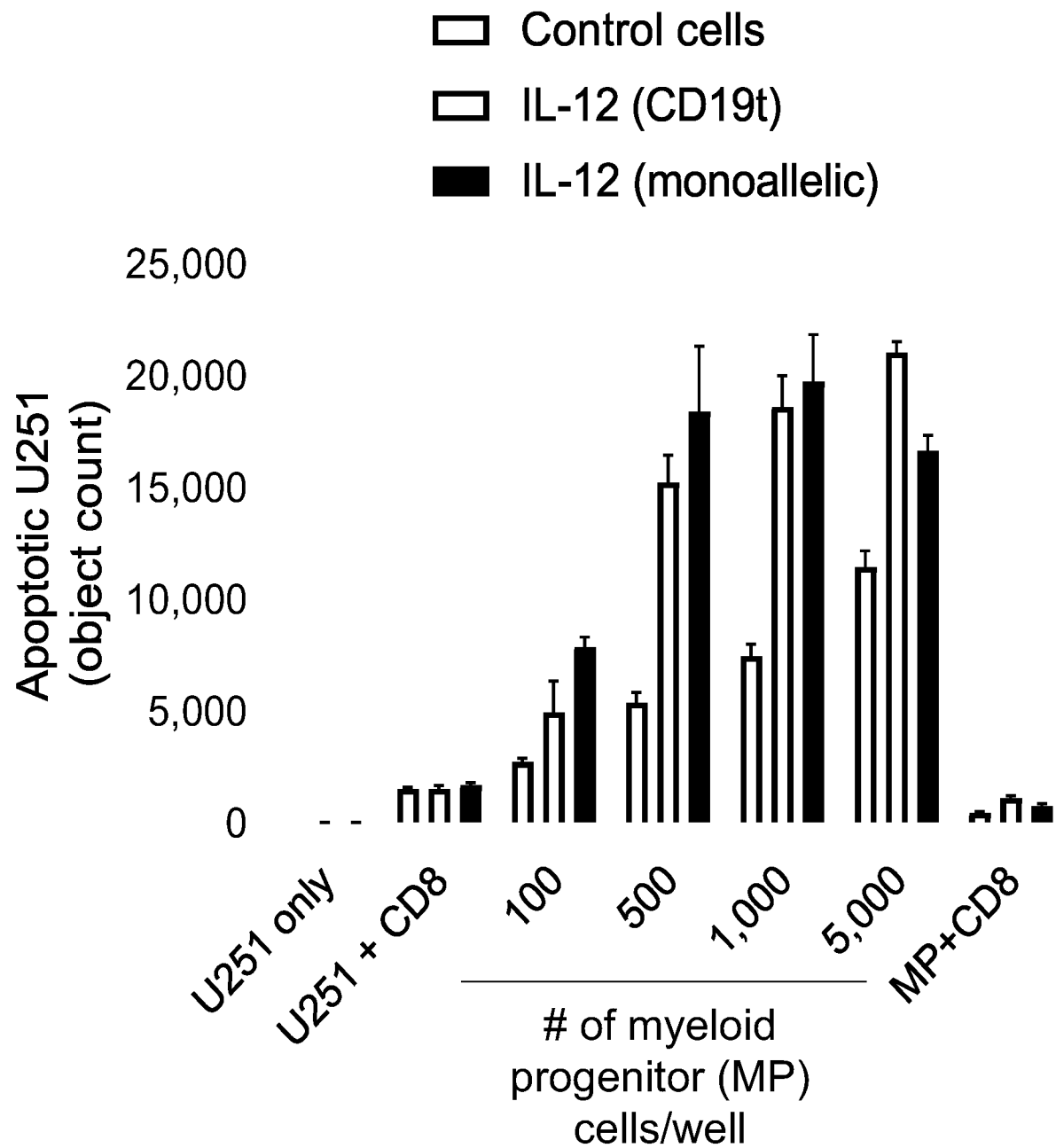


FIG. 7

8/24

**FIG. 8**

9/24

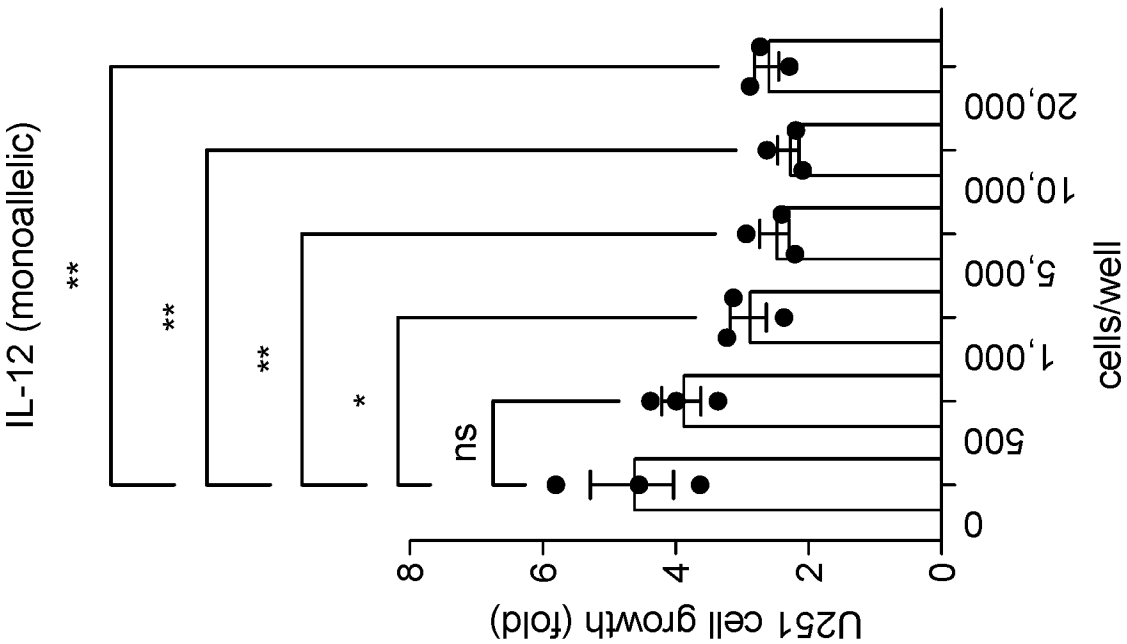


FIG. 9B

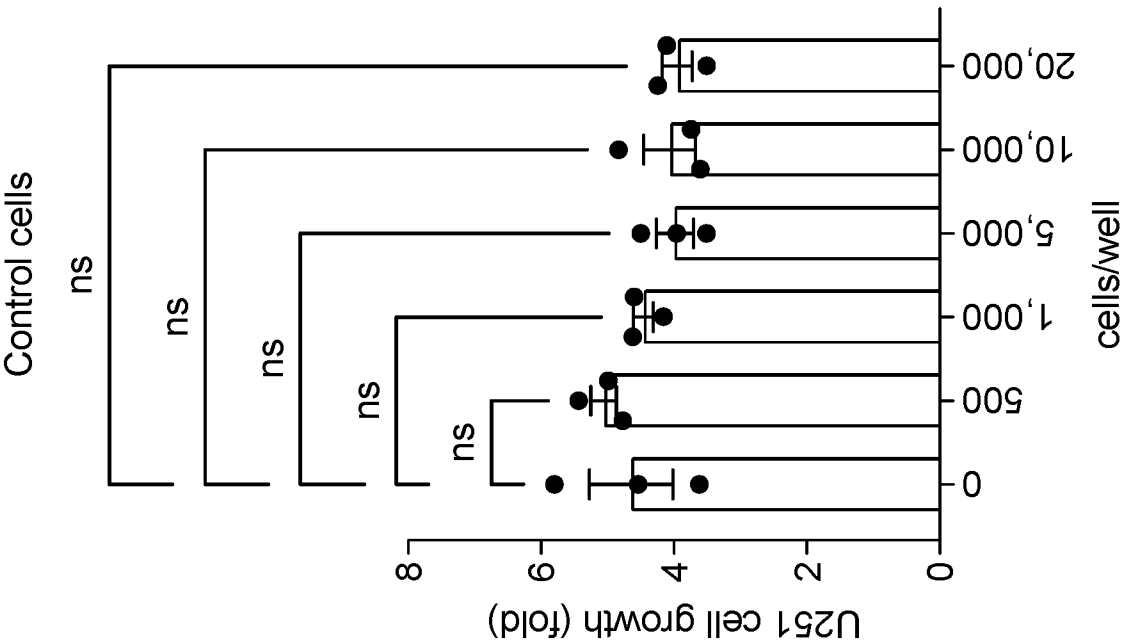
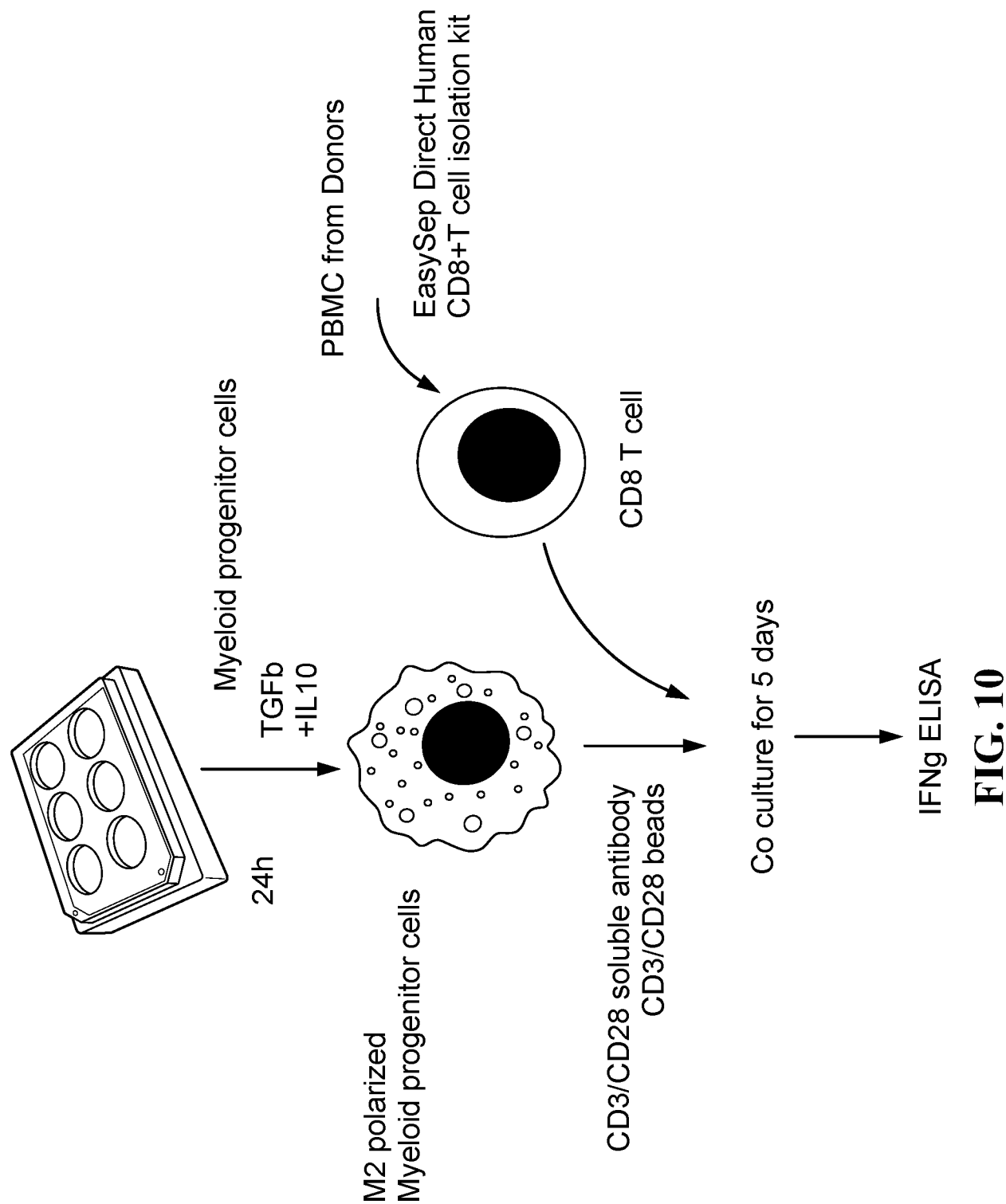


FIG. 9A

10/24



11/24

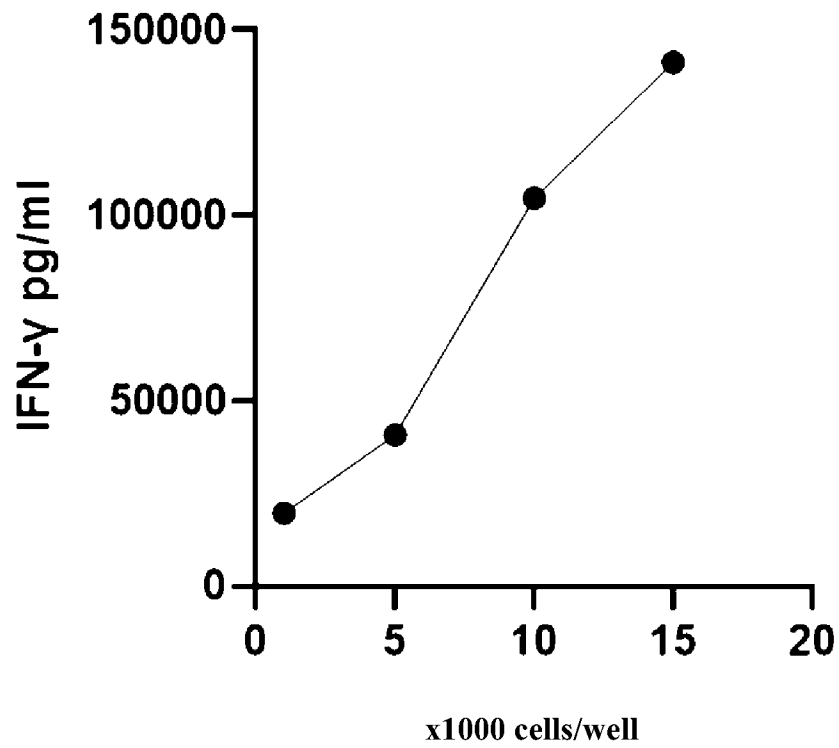


FIG. 11A

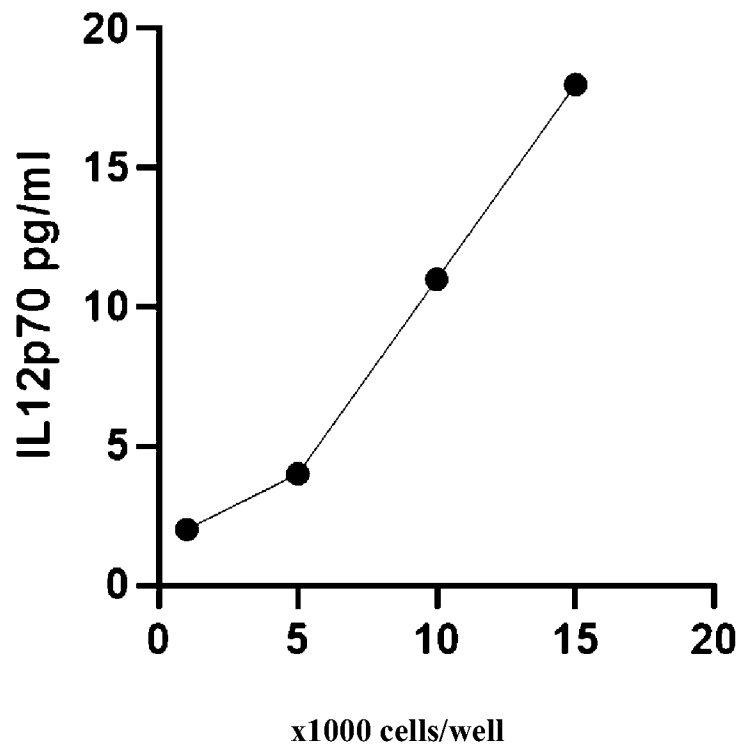


FIG. 11B

12/24

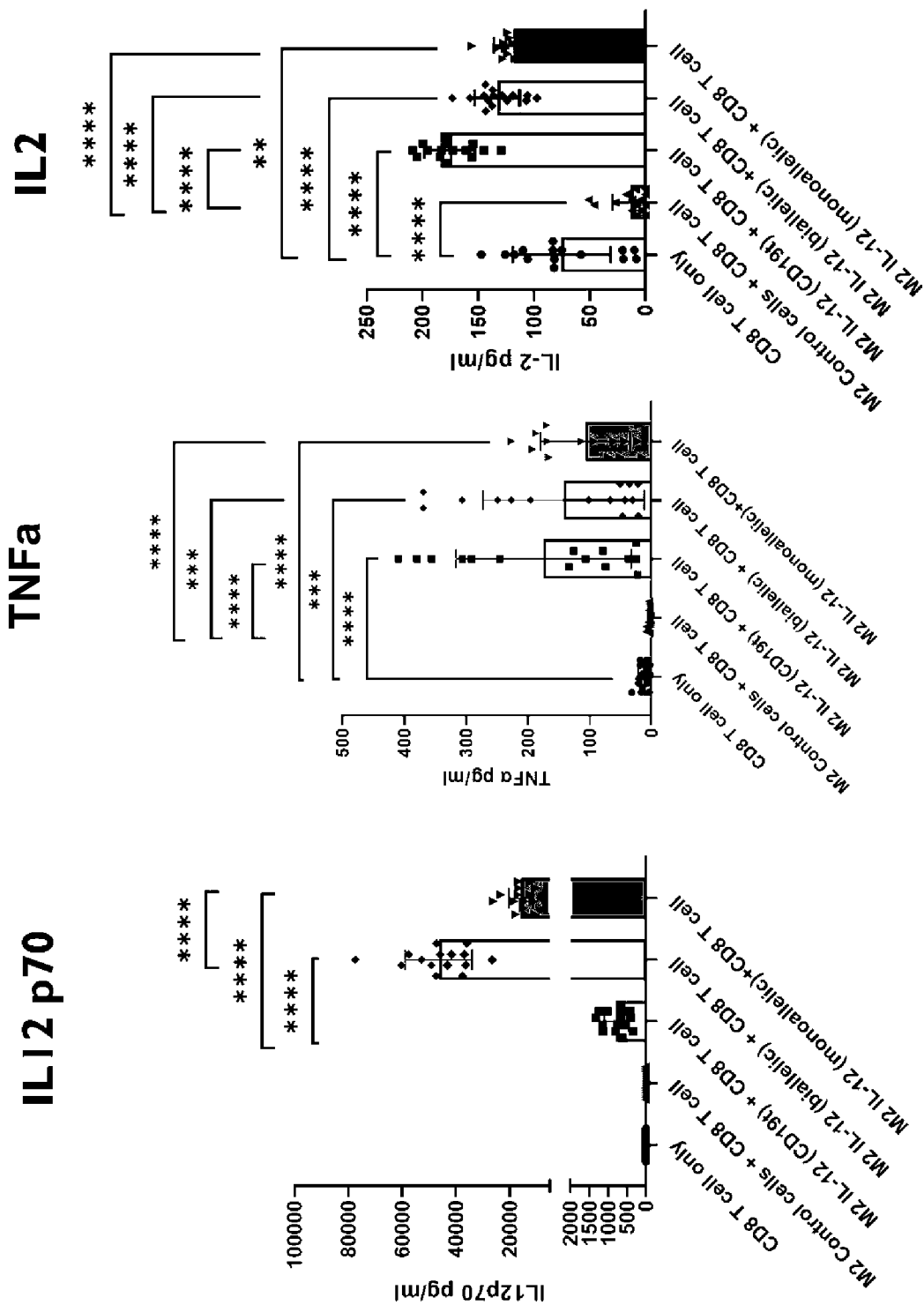


FIG. 12A

FIG. 12B

FIG. 12C

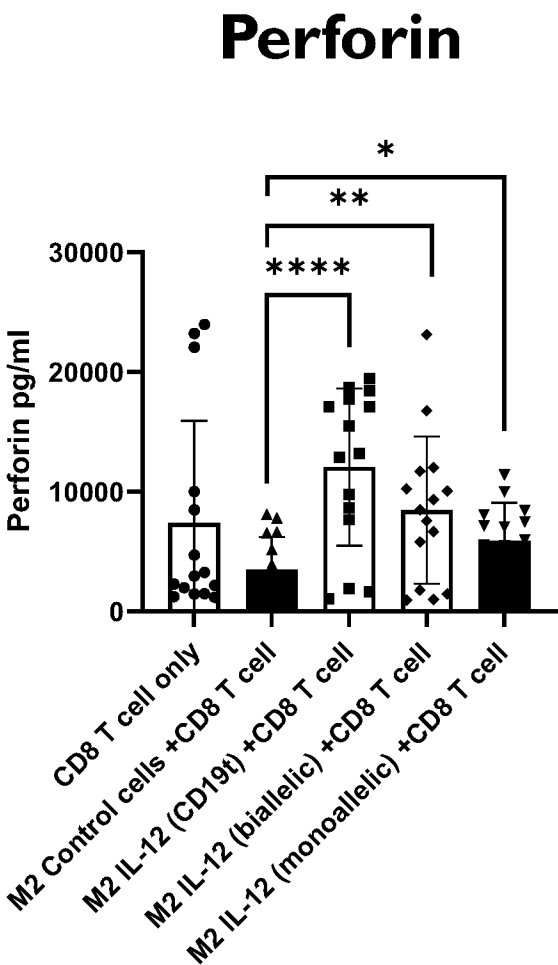


FIG. 12D

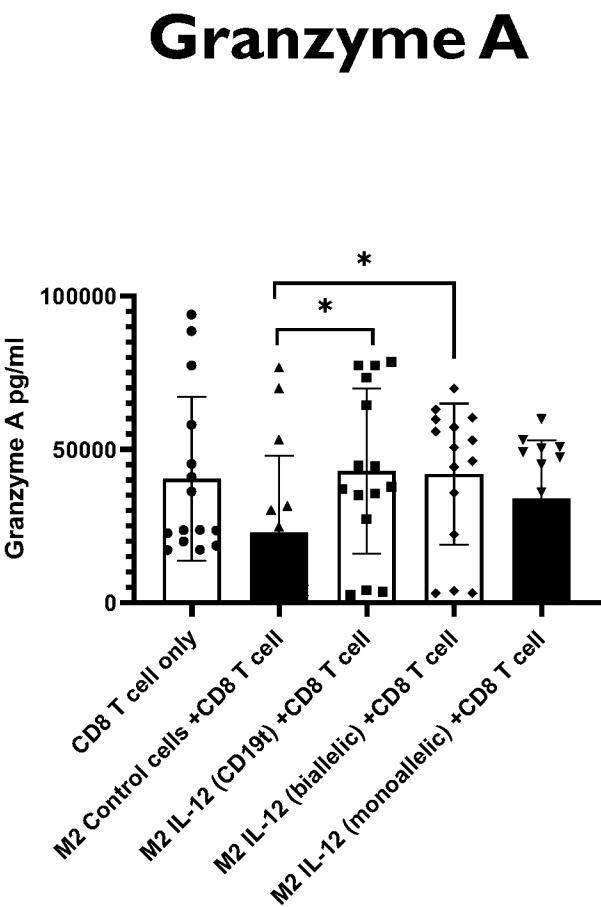


FIG. 12E

14/24

Granzyme B

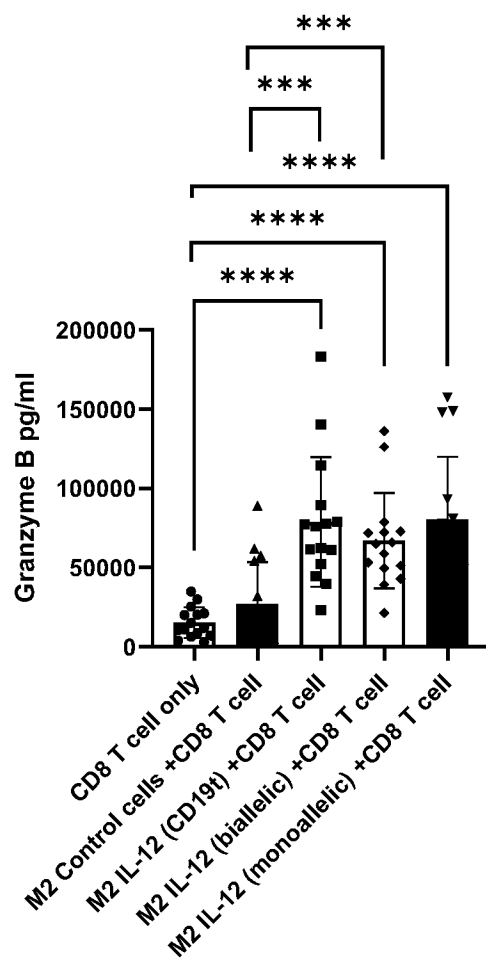


FIG. 12F

Granulysin

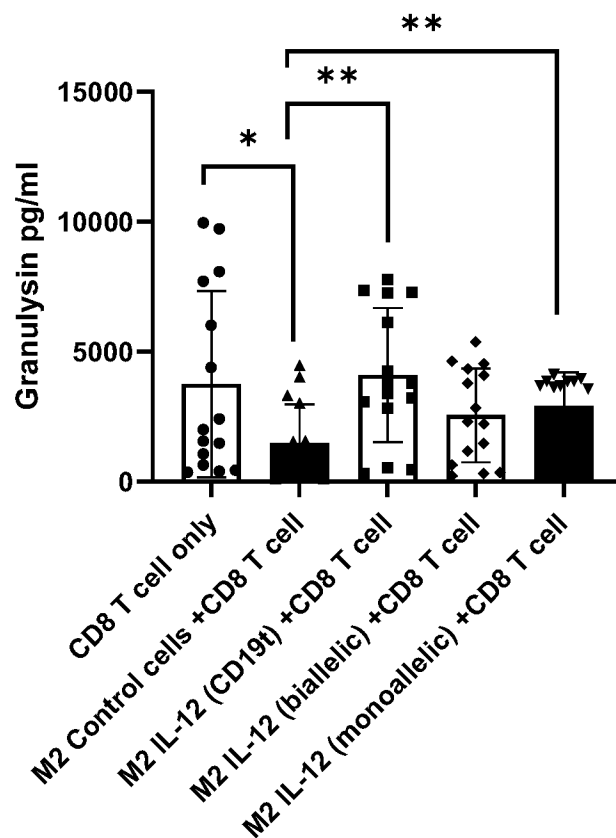
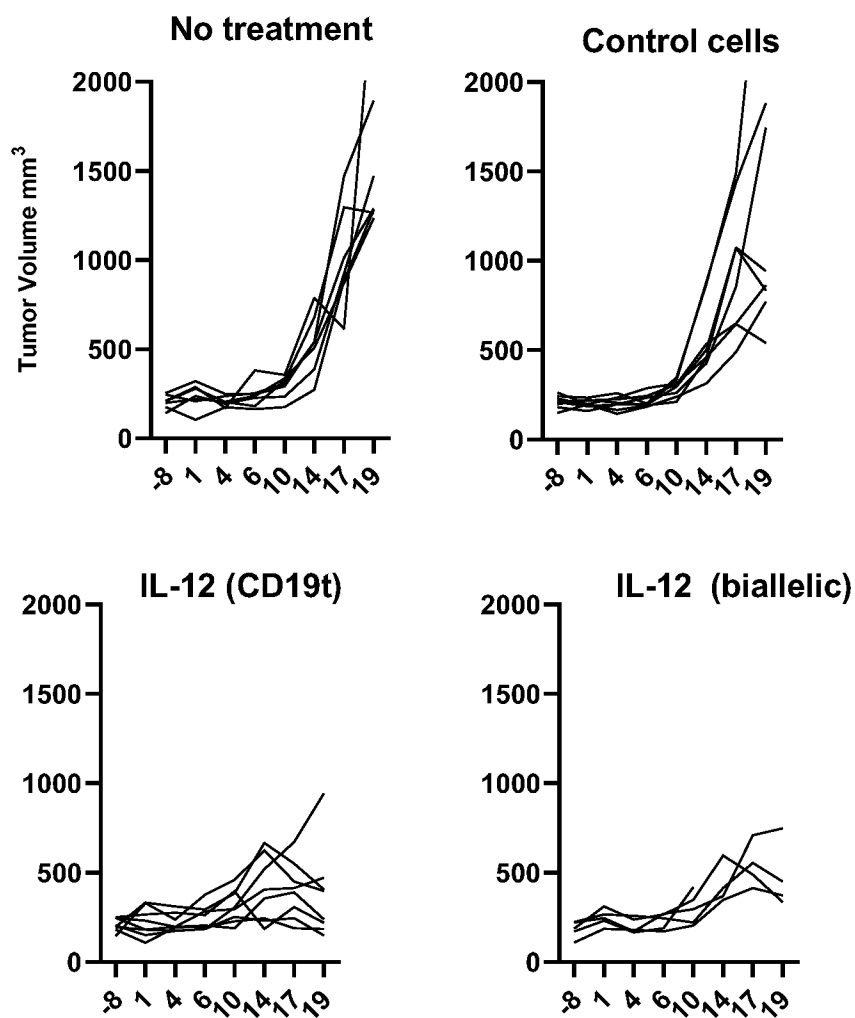
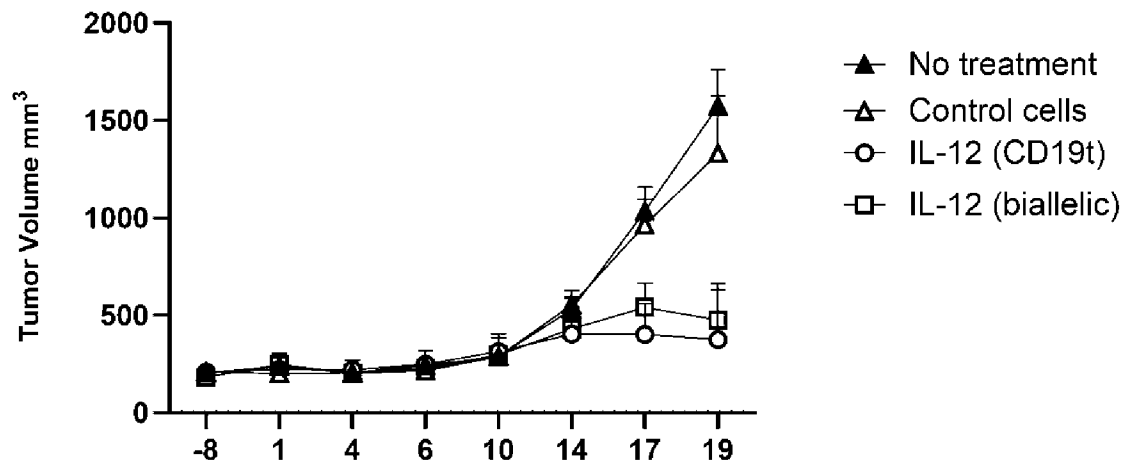


FIG. 12G

15/24

**FIG. 13**

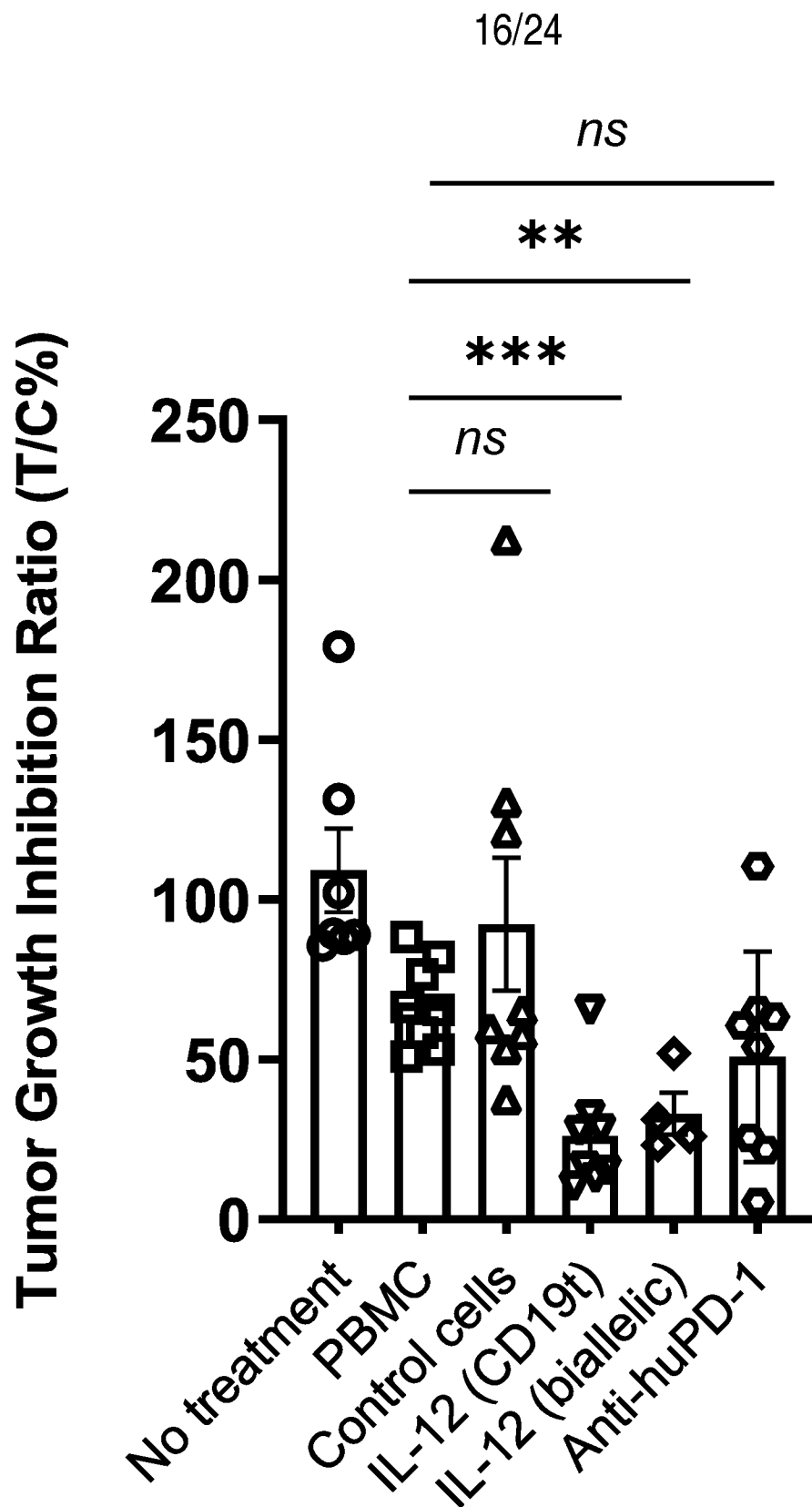
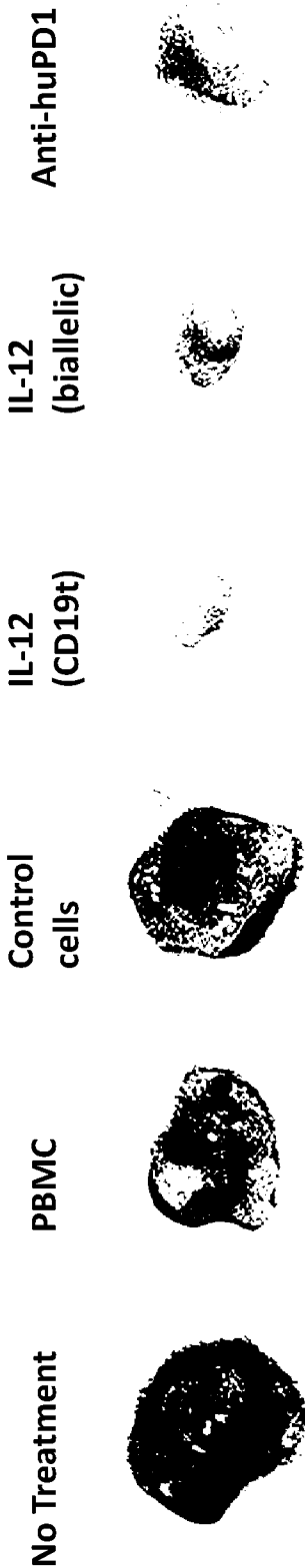


FIG. 14A



Representative images of harvested tumors, endpoint

FIG. 14B

18/24

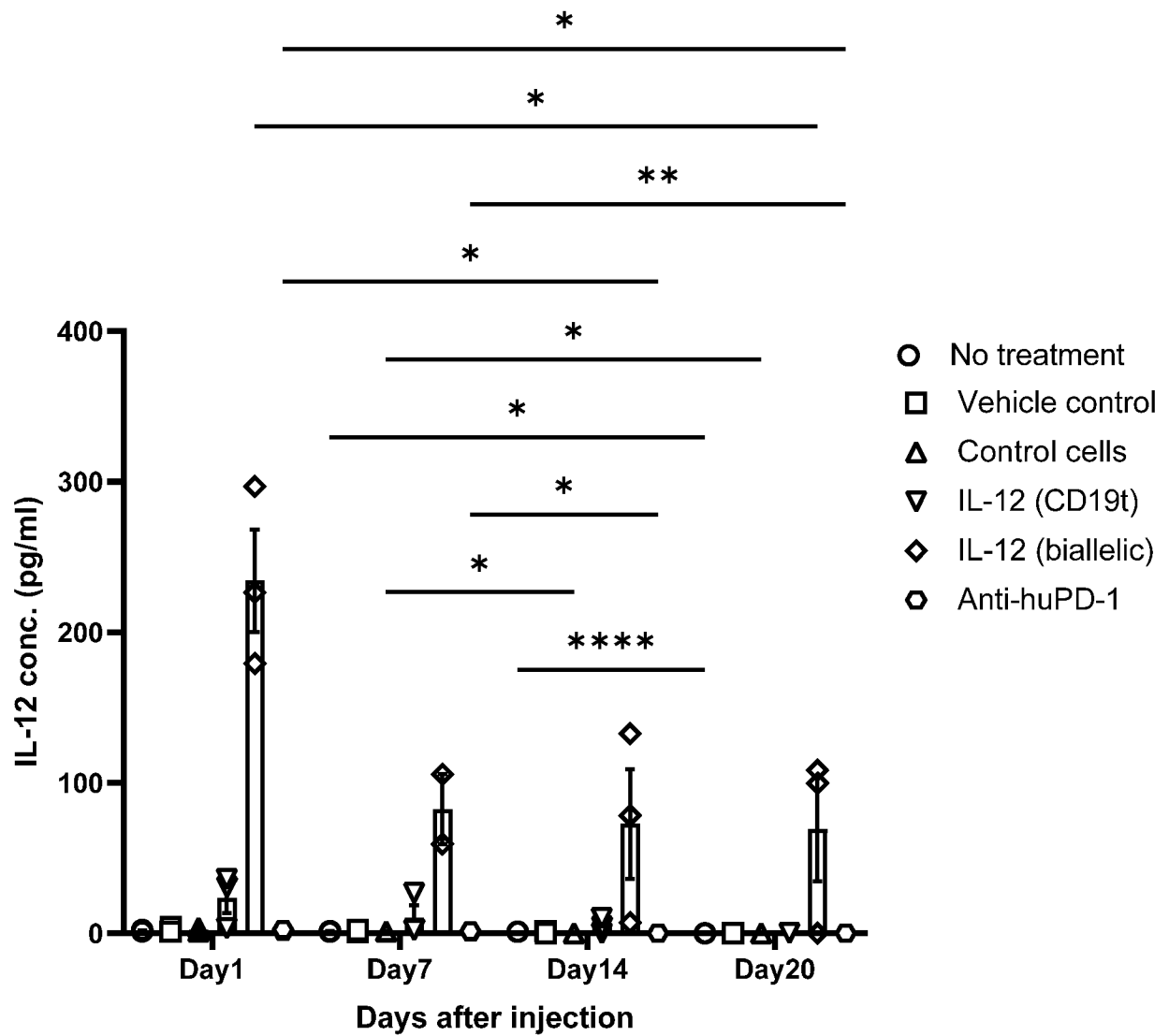


FIG. 15

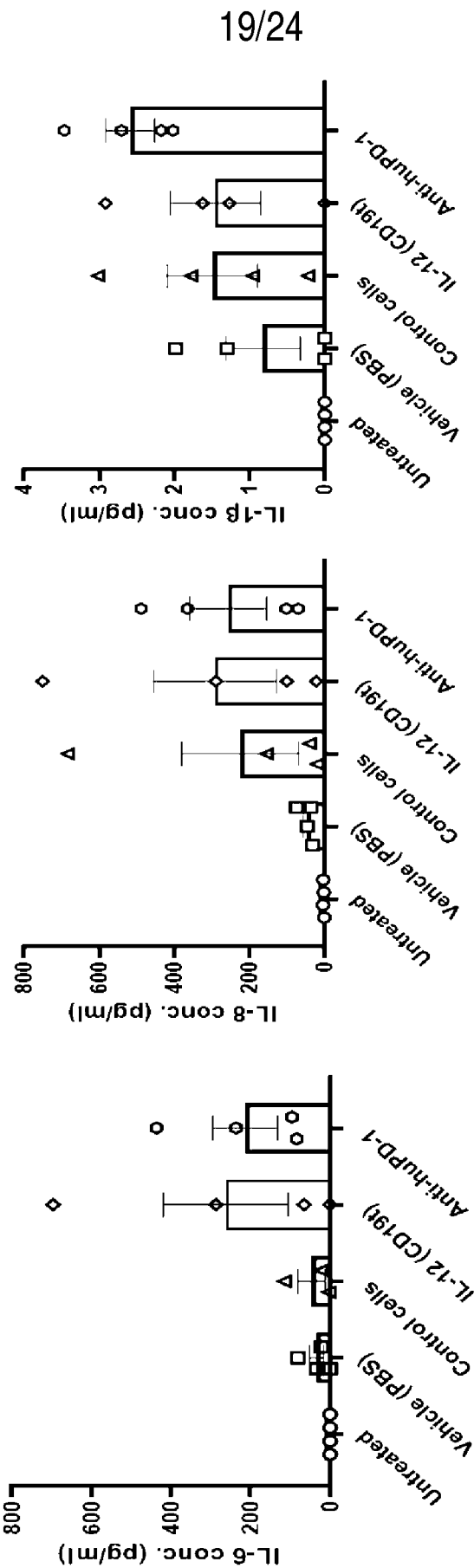


FIG. 16A

FIGS. 16B

FIGS. 16C

20/24

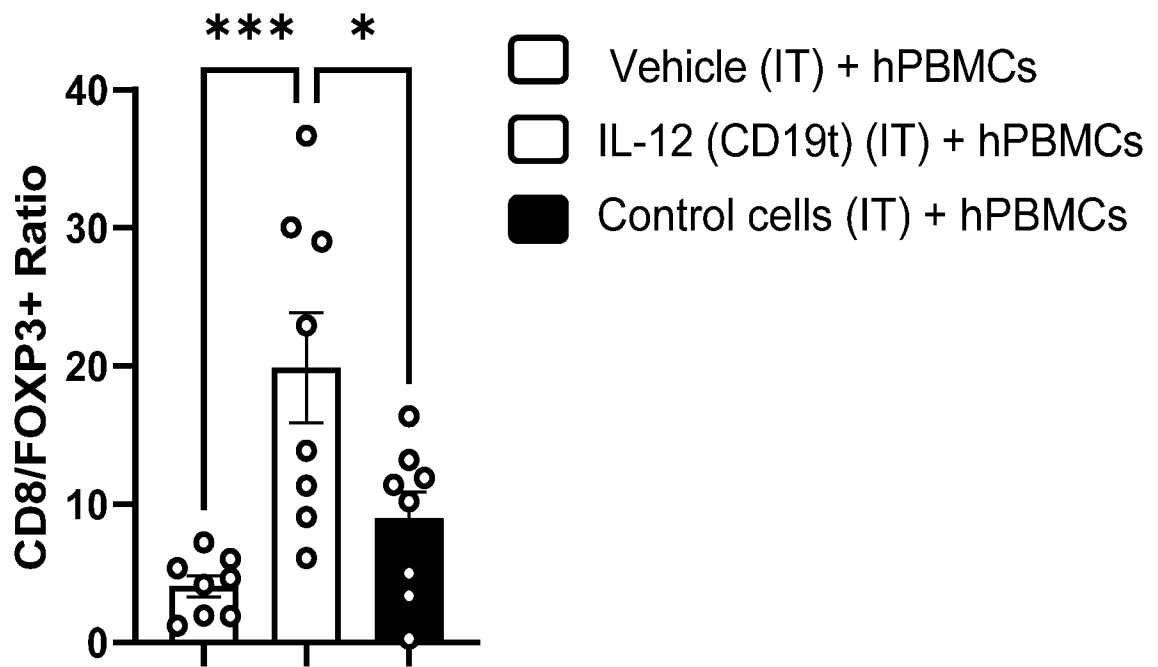


FIG. 17A

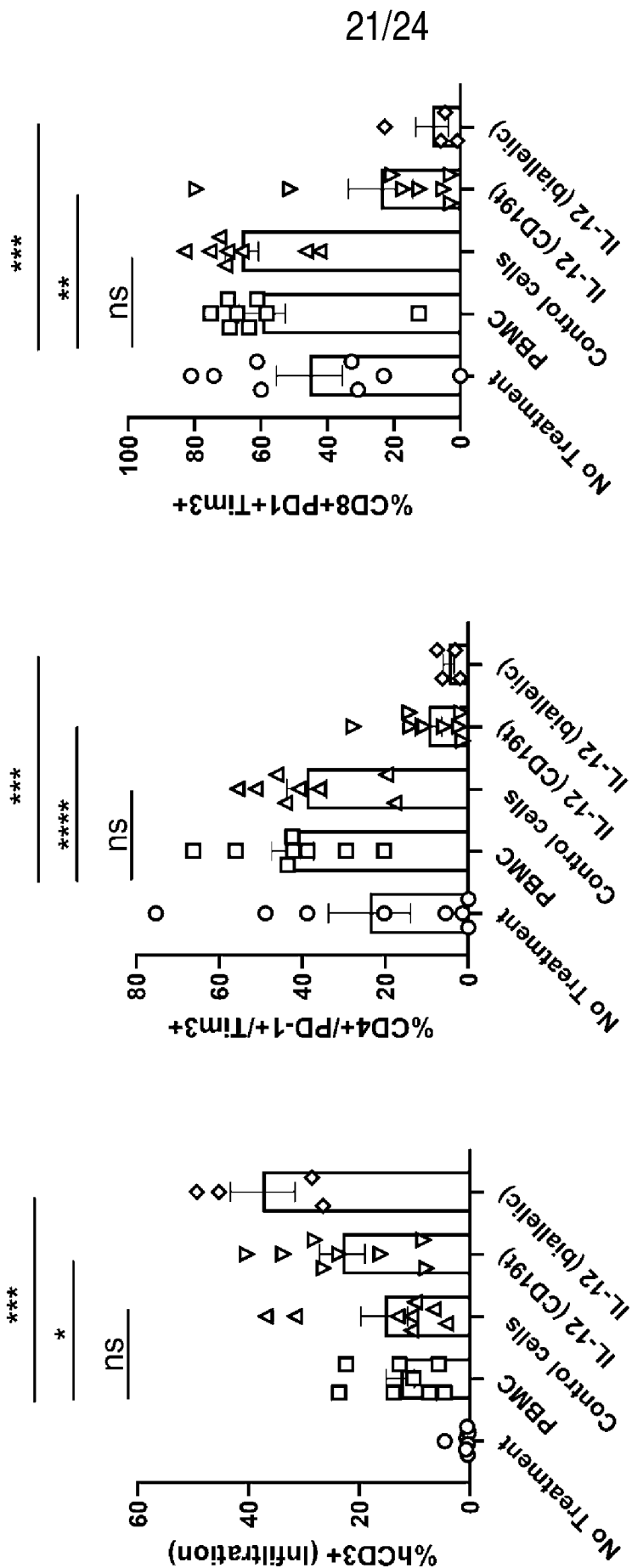


FIG. 17B

FIG. 17C

FIG. 17D

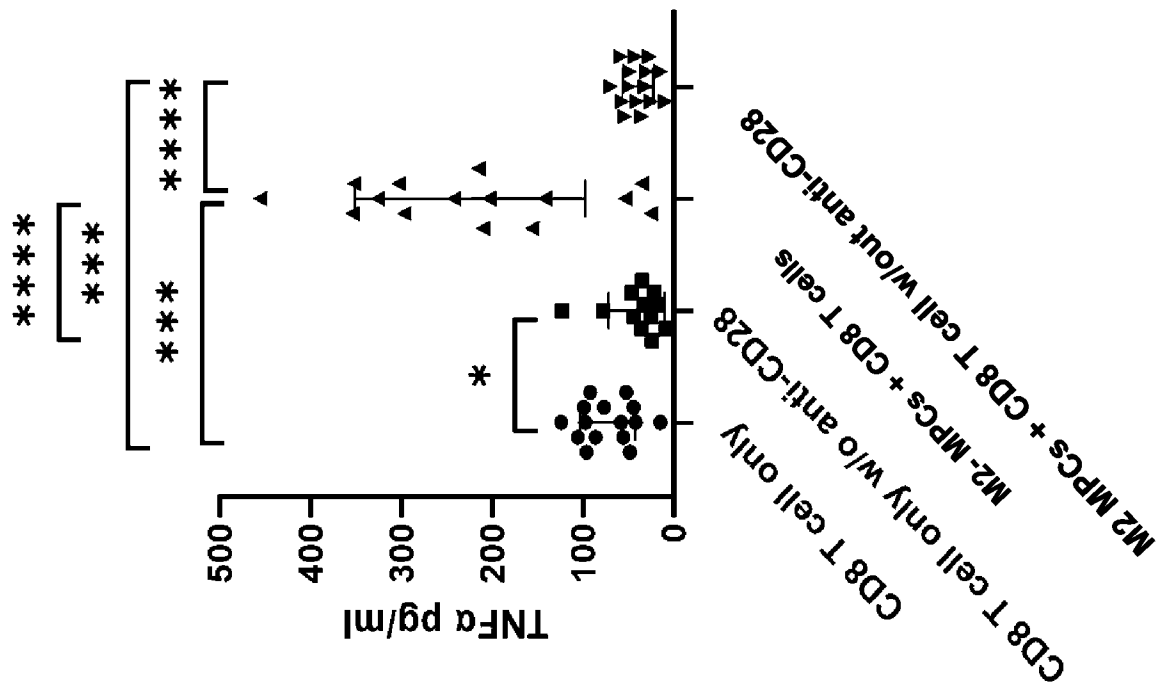


FIG. 18B

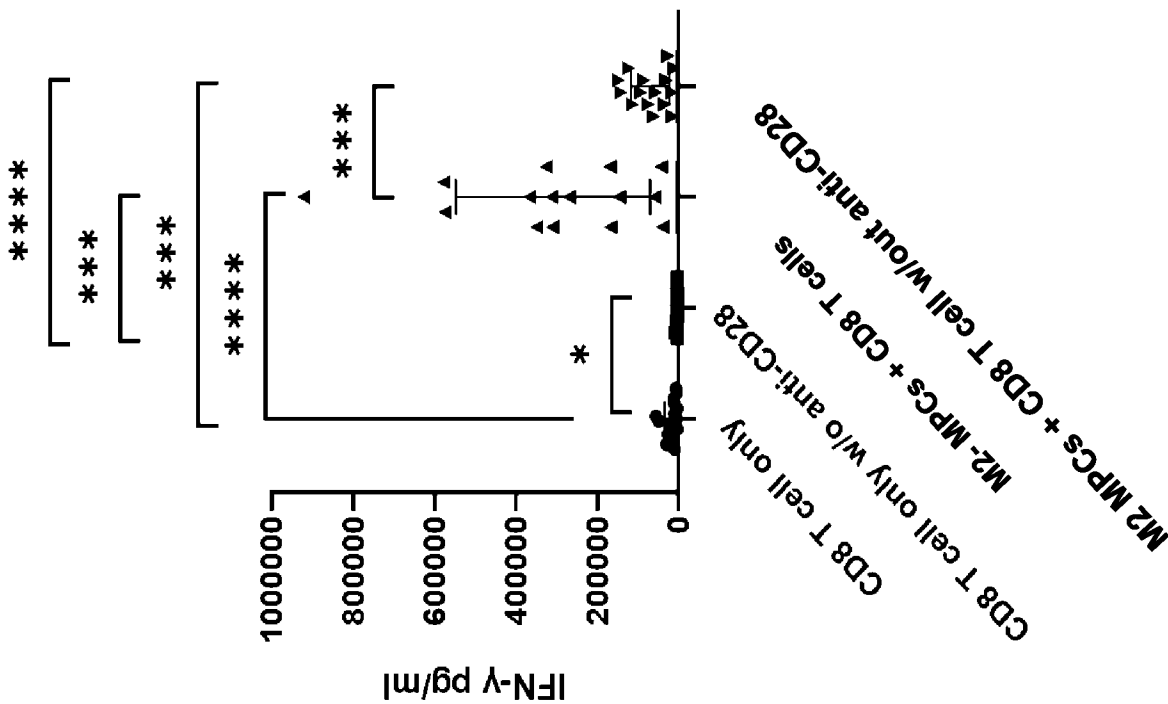
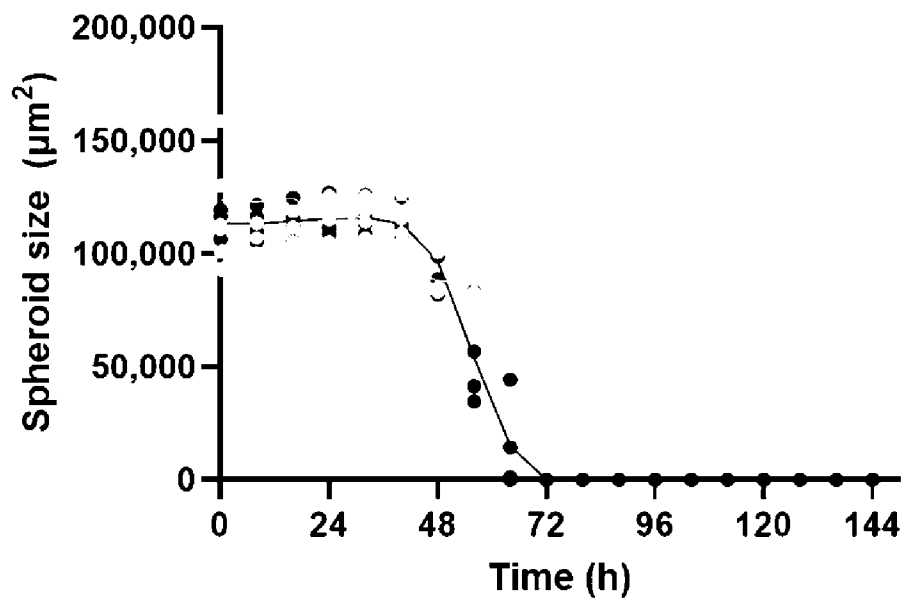
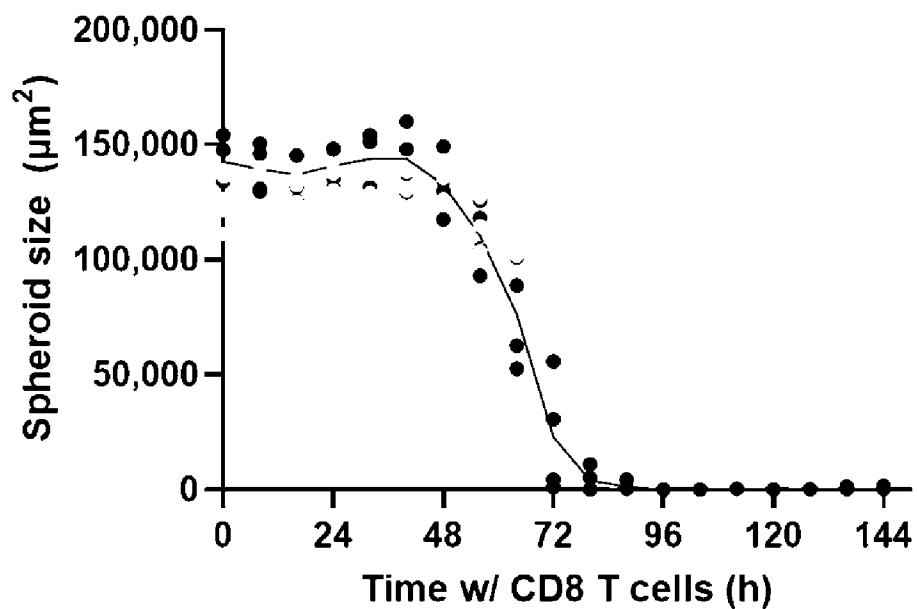


FIG. 18A

23/24

CD8T + aCD3 only**FIG. 19A****CD8T + aCD3 and aCD28****FIG. 19B**

24/24

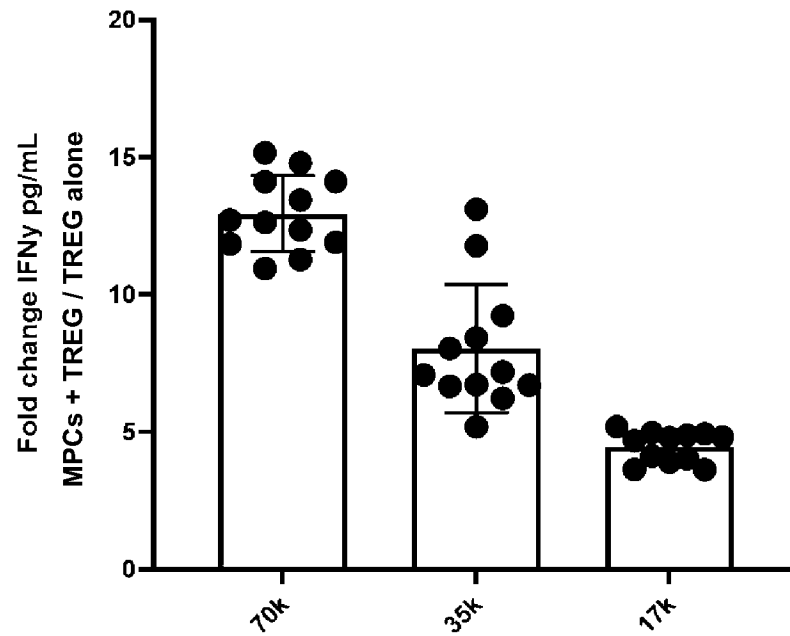


FIG. 20A

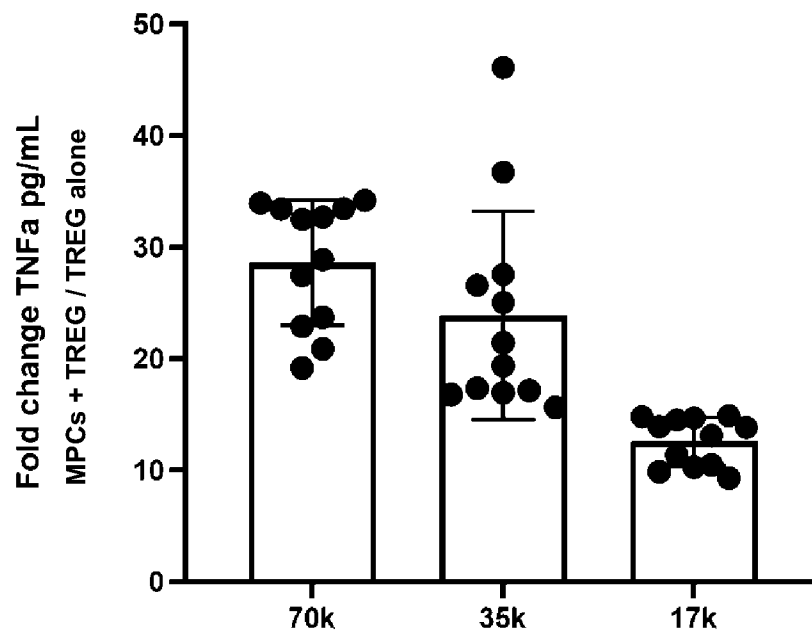


FIG. 20B