

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

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
14 May 2021 (14.05.2021)

(10) International Publication Number
WO 2021/092007 A1

(51) International Patent Classification:

A61K 35/17 (2015.01) C07K 14/725 (2006.01)
C07K 16/28 (2006.01)

(21) International Application Number:

PCT/US2020/058870

(22) International Filing Date:

04 November 2020 (04.11.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/930,221 04 November 2019 (04.11.2019) US

(71) Applicant: **VERASTEM, INC.** [US/US]; 117 Kendrick Street, Suite 500, Needham, MA 02494 (US).

(72) Inventors: **PACHTER, Jonathan, A.**; 2 Barley Lane, Wayland, MA 01778 (US). **COMA, Silvia**; 22 Birch Street, Needham, MA 02494 (US).

(74) Agent: **MCCARTY, Catherine, M.** et al.; Goodwin Procter LLP, 100 Northern Avenue, IP Docketing Dept./7th FL, Boston, MA 02210 (US).

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

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

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHODS OF MAKING CELLULAR THERAPIES

(57) Abstract: The present disclosure relates generally to methods of making immune effector cells, such as those engineered to express a Chimeric Antigen Receptor (CAR) including CAR.-T cells, and compositions comprising the same, for use in treating cancer.

WO 2021/092007 A1

METHODS OF MAKING CELLULAR THERAPIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Application No. 62/930,221, filed on November 4, 2019, the disclosure of which is hereby incorporated by
5 reference in its entirety for all purposes.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 2, 2020, is named VSM-064WO_ST25.txt and is 39,654
10 bytes in size.

FIELD OF THE DISCLOSURE

[0003] The present invention relates generally to methods of making immune effector cells, such as cells engineered to express a Chimeric Antigen Receptor (CAR) *e.g.*, CAR-T cells, and compositions comprising the same, for use in treating cancer.

15 BACKGROUND

[0004] Adoptive cell transfer (ACT) therapy with T-cells, especially with T-cells transduced with Chimeric Antigen Receptors (CARs, also referred to as CAR-T cells), is a promising treatment for multiple cancers.

[0005] The production of genetically-modified T cells is a complex process that starts with a
20 subject's material, obtained from leukapheresis, from which the engineered therapeutic T cells expressing a CAR are derived. Patient starting materials often display a high level of cell variability, varying greatly in cellular composition from patient to patient and even within a single disease state. Cellular impurities, including other classes of immune cells, can affect the yield of the desired cells. Poor cell yield, poor cell viability, or high levels of T cell exhaustion
25 can further contribute to manufacturing challenges. Additionally, diverse treatment histories of patients, state and stages of disease, and additional factors contribute to the heterogeneity of patient samples and further impact the cellular content of the starting material (Burger *et al.* 2014, Kaiser *et al.* 2015, and Ramos *et al.* 2009) Overall quality of the starting material can negatively impact the manufacturing process and quality of the end product.

[0006] Therefore, there exists a need for improved methods and processes for more consistent production of the CAR-expressing cell therapy product, to improve the manufacturing process, product quality, and maximize the therapeutic efficacy of the product.

SUMMARY

5 [0007] The present disclosure pertains to methods of making immune effector cells (*e.g.*, T cells, NK cells), such as those that can be engineered to express a chimeric antigen receptor (CAR), and compositions comprising the same.

[0008] Described herein are methods of making immune effector cells, such as those expressing chimeric antigen receptors (*e.g.*, T cells, NK cells), wherein the method comprises
10 administering a PI3K inhibitor and collecting a subjects' immune effector cells. In some embodiments, the immune effector cells are tumor infiltrating lymphocytes (TILs). In some embodiments, the method further comprises introducing a nucleic acid into the immune effector cells such that they express chimeric antigen receptors. In some embodiments, the method comprises providing a population of immune effector cells, administering a dual PI3K δ/γ
15 inhibitor to a subject, and introducing a nucleic acid into the immune effector cells suitable for expression of a CAR. In some embodiments, the immune effector cells are NK cells. In some embodiments, the immune effector cells are T cells.

[0009] In one embodiment, the immune effector cells (*e.g.*, T cells) express a chimeric antigen receptor comprising an antigen binding-fragment directed against a tumor antigen, a
20 hinge or transmembrane region, and an intracellular T-cell signaling component. In embodiments, the tumor antigen is selected from the list comprising CD19, BCMA, CD123, CD20, CD22, CD70, and CD37. In some embodiments, the tumor antigen is CD19.

[0010] In one embodiment, the population of immune effector cells are autologous to the subject who the cells will be administered to for treatment. In one embodiment, the population
25 of immune effector cells are allogeneic to the subject who the cells will be administered to for treatment. In one embodiment, the subject is a mammal. In some embodiments, the subject is a human.

[0011] In some embodiments, the PI3K inhibitor is a PI3K α inhibitor, PI3K β inhibitor, PI3K γ inhibitor, PI3K δ inhibitor, or dual PI3K δ/γ inhibitor. In some embodiments, the PI3K inhibitor is
30 chosen from tenalisib, duvelisib, idelalisib, copanlisib, IPI-549, CAL-130, BKM 120, GDC-0941, PX-866, GDC-0032, BAY 80-6946, BEZ235, BYL719, BGT-226, GDC-0980, GSK 2126458, PF-05212384, XL765, AS604850, AS252424, or XL147, or a combination thereof. In

some embodiments, the PI3K inhibitor is a dual PI3K δ/γ inhibitor. In some embodiments, the dual PI3K δ/γ inhibitor is duvelisib.

[0012] In one embodiment, the immune effector cells, *e.g.*, CAR cells described herein, *e.g.*, CD19 CAR cells described herein, treated with a dual PI3K δ/γ , have an increased proportion of early memory T cells or non-exhausted early memory T cells or both, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, CAR cells described herein, *e.g.*, CD19 CAR cells described herein, expanded for 3, 4, or 5 days, have an increased proportion of early memory T cells or non-exhausted early memory T cells or both, compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, a population of cells, *e.g.*, CD19 CAR cells described herein, expanded for up to 9 days, has a percentage of early memory T cells or non-exhausted early memory T cells or both of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70%, and optionally up to about 70% to 90%. In certain embodiments, the cell-surface markers comprise one or more of CD62L, CD45RA, CD27, CD28, CCR7, CD127, CD45RO, CD95, CD95 and II -2R β . In some embodiments, administration of a dual PI3K δ/γ inhibitor increases CD8⁺CD27⁺CD45RO^{dim/ne} T-cells. As used herein, CD protein expression on a cell population may be defined as ^{hi}, ^{mid} or ^{low} (alternatively ^{bright}, ^{mid} or ^{dim}).

[0013] In one aspect, the early memory T cells are CD27⁺ and/or CD45RO^{dim/ne}. In another aspect, the non-exhausted early memory T cells are PD-1 negative, CD27^{hi}, CCR7^{hi}, and/or CD45RO^{dim/neg}. In certain embodiments, administration of the dual PI3K δ/γ inhibitor increases CD27 expression in the T cell population compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ/γ inhibitor. In some embodiments, administration of a dual PI3K δ/γ inhibitor increases naïve CD4 and CD8 T-cells and central memory CD8⁺ T-cells. In some embodiments, markers on the immune effector cells are measured using methods known in the art, such as flow cytometry, to determine increased product after dual PI3K δ/γ exposure.

[0014] In some embodiments, following administration of a dual PI3K δ/γ inhibitor, the population of T cells has an increased amount of increased by increased by about 5%, about 10%, about 15%, about 20% ... about 90% or more (including all values and ranges in between) early memory T cells or non-exhausted early memory T cells compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ/γ inhibitor.

- [0015] In some embodiments, following administration of a dual PI3K δ / γ inhibitor, the population of T cells has an increased amount of about 5%, about 10%, about 15%, about 20% ... about 90% or more (including all values and ranges in between) CD8/CD4 positive T cells compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ / γ inhibitor. In certain embodiments, the increased amount is about 40% more CD8/CD4 positive T cells compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ / γ inhibitor.
- [0016] In some embodiments, administration of a dual PI3K δ / γ inhibitor decreases reliance on glycolysis and increases oxidative phosphorylation. In some embodiments, administration of a dual PI3K δ / γ inhibitor increases mRNA expression levels of proteins involved in the pentose phosphate pathway. In some embodiments, administration of a dual PI3K δ / γ inhibitor increases mRNA expression levels of autophagy pathway proteins. In some embodiments, administration of a dual PI3K δ / γ inhibitor increases fatty acid synthesis. In some embodiments, administration of a dual PI3K δ / γ inhibitor increases T-cell mitochondrial mass and mitochondrial respiration.
- [0017] In one embodiment, the method of making a population of Chimeric Antigen Receptor (CAR)-expressing immune effector cells, comprises providing a population of immune effector cells, wherein the immune effector cells are contacted with a nucleic acid encoding a CAR polypeptide and a dual PI3K δ / γ inhibitor; and maintaining the cells under conditions that allow expression of the CAR polypeptide, thereby making a population of CAR-expressing immune effector cells.
- [0018] In one embodiment, the method further comprises transducing a cell from the population of immune effector cells, *e.g.*, the population of T cells, with an expression vector comprising a nucleic acid encoding a CAR, *e.g.*, a CAR described herein, *e.g.*, a CD19 CAR described herein. In one embodiment, the expression vector is selected from the group consisting of a DNA, an RNA, a plasmid, a lentiviral vector, an adenoviral vector, or a retroviral vector. In one embodiment, cells from the population of immune effector cells, *e.g.*, the population of T cells, are transduced with vector once, *e.g.*, within one day after the population of immune effector cells are obtained from a blood sample from a subject. In one embodiment, the method further comprises generating a population of RNA-engineered cells transiently expressing exogenous RNA from the population of immune effector cells, *e.g.*, the population of T cells. In an embodiment, the method comprises introducing an *in vitro* transcribed RNA or a synthetic RNA into cells from the population, wherein the RNA comprises a nucleic acid encoding a CAR, *e.g.*, a CAR described herein, *e.g.*, a CD19 CAR described herein.

- [0019] In one embodiment, cells transduced with a nucleic acid encoding a CAR, *e.g.*, a CAR described herein, *e.g.*, a CD19 CAR described herein, are expanded, *e.g.*, by a method described herein. In one embodiment, the cells are expanded in culture for a period of several hours (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, 21 hours) to about 14 days (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days). In one embodiment, the cells are expanded for less than 12 hours. In one embodiment, the cells are expanded for 12 to 24 hours. In one embodiment, the cells are expanded for 1 to 3 days. In one embodiment, the cells are expanded for a period of 3 to 9 days. In one embodiment, the cells are expanded for a period of 4 to 9 days. In one embodiment, the cells are expanded for a period of 8 days or less, *e.g.*, 7, 6, 5, 4, or 3 days.
- 10 [0020] In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, are expanded in culture for 3 or 4 days, and the resulting cells are more potent than the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, are expanded in culture for 5 days, and the resulting cells are more potent than the same cells expanded in culture for 9 days under the same culture conditions.
- 15 Potency can be defined, *e.g.*, by various T cell functions, *e.g.* proliferation, target cell killing, cytokine production, activation, migration, or combinations thereof. In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, expanded for 3 or 4 days show at least a one, two, three or four fold increase in cells doublings upon antigen stimulation as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the
- 20 cells, *e.g.*, a CD19 CAR cell described herein, expanded for 5 days show at least a one, two, three or four fold increase in cells doublings upon antigen stimulation as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, the cells expressing a CD19 CAR described herein, are expanded in culture for 3 or 4 days, and the resulting cells exhibit higher proinflammatory cytokine production, *e.g.*, IFN- γ
- 25 and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, the cells expressing a CD19 CAR described herein, are expanded in culture for 5 days, and the resulting cells exhibit higher proinflammatory cytokine production, *e.g.*, IFN- γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment,
- 30 the cells, *e.g.*, a CD19 CAR cell described herein, expanded for 3 or 4 days show at least a one, two, three, four, five, tenfold or more increase in pg/ml of proinflammatory cytokine production, *e.g.*, IFN- γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, expanded for 5 days show at least a one, two, three, four, five, tenfold or more

Attorney Docket No.: VSM-064WO

increase in pg/ml of proinflammatory cytokine production, *e.g.*, IFN- γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, expanded for 3, 4, or 5 days show at least as high cytokine production in pg/ml, or at least a one, two, three, four, five, ten-
5 fold or more or more than ten-fold increase in pg/ml of cytokine production, *e.g.*, IL2, IFN- γ , GM-CSF, TNF- α , IL-1 β , IL4, IL5, IL6, IL8, or IL10 levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions.

[0021] In one embodiment, the cells are expanded by culturing the cells in the presence of an agent that stimulates a CD3/TCR complex associated signal, and a ligand that stimulates a
10 costimulatory molecule on the surface of the cells, *e.g.*, as described herein. In one embodiment, the agent is a bead conjugated with anti-CD3 antibody, or an antigen-binding fragment thereof, and/or anti-CD28 antibody, or an antigen-binding fragment thereof.

[0022] In one embodiment, the cells are expanded in an appropriate culture media (*e.g.*, a culture media described herein) that may, optionally, contain one or more (*e.g.*, 1, 2, 3, 4, or 5 or
15 more) factors for proliferation and/or cellular viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, IL-21, TGF β , and TNF- α or any other additives for the growth of cells.

[0023] In one embodiment, the immune effector cells are contacted with the nucleic acid encoding a CAR polypeptide prior to contact with the dual PI3K δ/γ inhibitor. In one
20 embodiment, the immune effector cells are contacted with the nucleic acid encoding a CAR polypeptide subsequent to contact with the dual PI3K δ/γ inhibitor. In one embodiment, the immune effector cells are contacted with the nucleic acid encoding a CAR polypeptide concurrently contact with the dual PI3K δ/γ inhibitor. In some embodiments, the dual PI3K δ/γ inhibitor is duvelisib.

25 [0024] In one aspect, disclosed is a method of treating a subject having cancer, the method comprising (i) dosing with a dual PI3K δ/γ inhibitor, and (ii) administering to the subject a therapeutically effective amount of CAR-expressing immune effector cells. In some embodiments, the dual PI3K δ/γ inhibitor is administered at a dose sufficient to increase populations of early memory CAR-T cells in the subject. In some embodiments, the subject is
30 administered a dual PI3K δ/γ inhibitor for 1, 2, 3, 4, or 5 or more weeks. In some embodiments, the subject is administered a dual PI3K δ/γ inhibitor q.d., b.i.d., or q.i.d.. In certain embodiments,

Attorney Docket No.: VSM-064WO

the subject is administered a dual PI3K δ / γ inhibitor b.i.d. In certain embodiments, the subject is administered a dual PI3K δ / γ inhibitor b.i.d. at 25 mg.

- [0025] In one embodiment, the dual PI3K δ / γ inhibitor is administered prior to administration of CAR-expressing immune effector cells. In one embodiment, the dual PI3K δ / γ inhibitor is administered concurrently with administration of CAR-expressing immune effector cells. In one embodiment, the dual PI3K δ / γ inhibitor is administered subsequent to administration of CAR-expressing immune effector cells. In some embodiments, the dual PI3K δ / γ inhibitor is duvelisib. In some embodiments, the CAR-expressing immune effector cells are CAR-T cells. In some embodiments, the CAR-T cells are CD19 CAR-T cells.
- 10 [0026] In embodiments, the CD19 CAR-T cells are administered in a therapeutic product, *e.g.*, tisagenlecleucel, axicabtagene ciloleucel, lisocabtagene maraleucel, or brexucabtagene autoleucel. In embodiments, the CD19 CAR-T cells are administered in a therapeutic product, *e.g.*, tisagenlecleucel, axicabtagene ciloleucel, or lisocabtagene maraleucel. In one embodiment, the therapeutic product is tisagenlecleucel. In one embodiment, the therapeutic product is
- 15 axicabtagene ciloleucel. In one embodiment, the therapeutic product is lisocabtagene maraleucel. In one embodiment, the therapeutic product is brexucabtagene autoleucel. In some embodiments, duvelisib is administered at a dose sufficient to increase populations of early memory CAR-T cells in the subject. In some embodiments, the method is used in combination with a second cancer treatment. In some embodiments, the second cancer treatment is a
- 20 chemotherapy. In some embodiments, the efficacy, safety, and duration of the subject's response of the CAR-T cell therapy is further assessed.

- [0027] In another aspect, provided herein is reaction mixture comprising a population of immune effector cells, wherein a plurality of the cells of the population in the reaction mixture comprise a vector comprising a nucleic acid sequence encoding a CAR, and wherein the reaction
- 25 mixture further comprises a dual PI3K δ / γ inhibitor. In a preferred embodiment, the immune effector cells are T cells. In some embodiments, the dual PI3K δ / γ inhibitor is duvelisib. In some embodiments, the reaction mixture comprises CAR-T cells and duvelisib.

- [0028] In embodiments, the reaction mixture comprises a population of immune effector cells, *e.g.*, T cells. The reaction mixture may further comprise a buffer or other reagent, *e.g.*, a
- 30 PBS containing solution. In one embodiment, the reaction mixture further comprises an agent that activates and/or expands to cells of the population, *e.g.*, an agent that stimulates a CD3/TCR complex associated signal and/or a ligand that stimulates a costimulatory molecule on the

Attorney Docket No.: VSM-064WO

surface of the cells, *e.g.*, as described herein. In one embodiment, the agent is a bead conjugated with anti-CD3 antibody, or a fragment thereof, and/or anti-CD28 antibody, or an antigen-binding fragment thereof. In one embodiment, the reaction mixture further comprises one or more (*e.g.*, 2, 3, 4, or 5) factor for proliferation and/or viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, IL-21, TGF- β , and TNF- α or any other additives for the growth of cells. In one embodiment, the reaction mixture further comprises IL-15 and/or IL-7. In one embodiment, the reaction mixture further comprises a cryoprotectant or stabilizer such as, *e.g.*, a saccharide, an oligosaccharide, a polysaccharide and a polyol (*e.g.*, trehalose, mannitol, sorbitol, lactose, sucrose, glucose and dextran), salts and crown ethers.

[0029] In some embodiments, a plurality of the cells of the population in the reaction mixture comprise a nucleic acid molecule, *e.g.*, a nucleic acid molecule described herein, that comprises a CAR encoding sequence, *e.g.*, a CD19 CAR encoding sequence, *e.g.*, as described herein. In one embodiment, a plurality of the cells of the population in the reaction mixture comprise a vector comprising a nucleic acid sequence encoding a CAR, *e.g.*, a CAR described herein, *e.g.*, a CD19 CAR described herein. In one embodiment, the vector is a vector described herein, *e.g.*, a vector selected from the group consisting of a DNA, a RNA, a plasmid, a lentivirus vector, adenoviral vector, or a retrovirus vector.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] FIG. 1 depicts concentration curves of whole blood stimulated with LPS and treated with PI3K inhibitors Duvelisib, Idelalisib, IPI-549, Umbralisib, and Tenalisib.

[0031] FIG. 2 depicts concentration curves of whole blood stimulated with fMLP and treated with PI3K inhibitors Duvelisib, Idelalisib, IPI-549, Umbralisib, and Tenalisib.

[0032] FIG. 3 shows T cell expansion following treatment with duvelisib (FIG. 3A) or idelalisib (FIG. 3B).

[0033] FIG. 4 shows expression of T cell markers TIM3 (FIG. 4A), CD8 (FIG. 4B), and LAG3 (FIG. 4C) as determined by flow cytometry.

[0034] FIG. 5 shows the proportions of T cell subtypes following standard culture (FIG. 5A) or treatment with duvelisib (FIG. 5B).

[0035] FIG. 6 depicts effect of duvelisib on CD8:CD4 CAR T ration (FIG. 6A) and CAR T-mediated cytotoxicity (FIG. 6B).

[0036] FIG. 7 demonstrates inhibition of both PI3K- δ (FIG. 7A) and PI3K- γ (FIG. 7B) activities in follicular lymphoma patients.

DETAILED DESCRIPTION

5 [0037] The invention provides to methods of making immune effector cells (*e.g.*, T cells, NK cells), such as those engineered to express a Chimeric Antigen Receptor (CAR), comprising treatment of the cells and/or patient with a PI3K-inhibitor, and compositions comprising the same, for use in treating cancer. The invention also provides pharmaceutical compositions comprising such immune effector cells, and therapeutic methods using such immune effector
10 cells and pharmaceutical compositions, for purposes such as treating cancer. Various aspects of the invention are set forth below in sections; however, aspects of the invention described in one particular section are not to be limited to any particular section.

[0038] To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

15 [0039] The terms “a” and “an” as used herein mean “one or more” and include the plural unless the context is inappropriate. The term "about" when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of +20% or in some instances +10%, or in some instances +5%, or in some instances +1%, or in some instances +0.1% from the specified value, as such variations are appropriate to perform the disclosed
20 methods.

[0040] The term "Chimeric Antigen Receptor" or alternatively a "CAR" refers to a set of polypeptides, typically two in the simplest embodiments, which when in an immune effector cell, provides the cell with specificity for a target cell, typically a cancer cell, and with intracellular signal generation. In some embodiments, a CAR comprises at least an extracellular
25 antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain (also referred to herein as "an intracellular signaling domain") comprising a functional signaling domain derived from a stimulatory molecule and/or costimulatory molecule as defined below. In some embodiments, the set of polypeptides are in the same polypeptide chain (*e.g.*, comprise a chimeric fusion protein). In some embodiments, the set of polypeptides are not contiguous with
30 each other, *e.g.*, are in different polypeptide chains. In some embodiments, the set of polypeptides include a dimerization switch that, upon the presence of a dimerization molecule, can couple the polypeptides to one another, *e.g.*, can couple an antigen binding domain to an

intracellular signaling domain. In one embodiment, the stimulatory molecule of the CAR is the zeta chain associated with the T cell receptor complex. In one aspect, the cytoplasmic signaling domain comprises a primary signaling domain (*e.g.*, a primary signaling domain of CD3-zeta). In one embodiment, the cytoplasmic signaling domain further comprises one or more functional signaling domains of at least one costimulatory molecule as defined below. In one embodiment, the costimulatory molecule is a costimulatory molecule described herein, *e.g.*, 4-1BB (*i.e.*, CD137), CD27, ICOS, and/or CD28. In one embodiment, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain of a stimulatory molecule. In one embodiment, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain of a co-stimulatory molecule and a functional signaling domain of a stimulatory molecule. In one embodiment, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising two functional signaling domains of one or more co-stimulatory molecule(s) and a functional signaling domain of a stimulatory molecule. In one embodiment, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising at least two functional signaling domains of one or more co-stimulatory molecule(s) and a functional signaling domain of a stimulatory molecule. In one embodiment, the CAR comprises an optional leader sequence at the amino-terminus (N-terminus) of the CAR fusion protein. In one embodiment, the CAR further comprises a leader sequence at the N-terminus of the extracellular antigen binding domain, wherein the leader sequence is optionally cleaved from the antigen binding domain (*e.g.*, an scFv) during cellular processing and localization of the CAR to the cellular membrane.

[0041] As used herein, “PI3K” refers to phosphoinositide 3-kinase, also called phosphatidylinositol 3-kinases in the art. The class I isoforms are PI3K α , PI3K β , PI3K δ , and PI3K γ . As used herein, PI3K α and PI3K α are used interchangeably, PI3K β and PI3K β are used interchangeably, PI3K δ and PI3K δ are used interchangeably, and PI3K γ and PI3K γ are used interchangeably.

[0042] A “dual PI3K δ / γ inhibitor”, as used herein, refers to a PI3K inhibitor that can reach the IC₅₀ from whole blood assays for both PI3K-delta and PI3K-gamma at clinically achievable plasma exposures.

[0043] A CAR that comprises an antigen binding domain (*e.g.*, an scFv, or TCR) that targets a specific tumor antigen X, such as those described herein, is also referred to as XCAR. For example, a CAR that comprises an antigen binding domain that targets CD19 is referred to as CD19CAR. The term "signaling domain" refers to the functional portion of a protein which acts
5 by transmitting information within the cell to regulate cellular activity via defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers. The term "antibody," as used herein, refers to a protein, or polypeptide sequence derived from an immunoglobulin molecule which specifically binds with an antigen.

[0044] Antibodies can be polyclonal or monoclonal, multiple or single chain, or intact
10 immunoglobulins, and may be derived from natural sources or from recombinant sources. Antibodies can be tetramers of immunoglobulin molecules. The term "antibody fragment" refers to at least one portion of an antibody, that retains the ability to specifically interact with (*e.g.*, by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')₂, Fv fragments,
15 scFv antibody fragments, disulfide-linked Fvs (sdFv), a Fd fragment consisting of the VH and CHI domains, linear antibodies, single domain antibodies such as sdAb (either VL or VH), camelid V_{HH} domains, multi-specific antibodies formed from antibody fragments such as a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region, and an isolated CDR or other epitope binding fragments of an antibody. An antigen binding
20 fragment can also be incorporated into single domain antibodies, maxibodies, minibodies, nanobodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (see, *e.g.*, Hollinger and Hudson, Nature Biotechnology 23: 1126-1136, 2005). Antigen binding fragments can also be grafted into scaffolds based on polypeptides such as a fibronectin type III (Fn3)(see U.S. Patent No.: 6,703,199, which describes fibronectin polypeptide minibodies).

[0045] The term "scFv" refers to a fusion protein comprising at least one antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked, *e.g.*, via a synthetic linker, *e.g.*, a short flexible polypeptide linker, and capable of being expressed as a single chain polypeptide, and wherein the scFv retains the
30 specificity of the intact antibody from which it is derived. Unless specified, as used herein an scFv may have the VL and VH variable regions in either order, *e.g.*, with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise VL-linker-VH or may comprise VH-linker-VL.

Attorney Docket No.: VSM-064WO

- [0046] The portion of a CAR comprising an antibody or antibody fragment thereof may exist in a variety of forms where the antigen binding domain is expressed as part of a contiguous polypeptide chain including, for example, a single domain antibody fragment (sdAb), a single chain antibody (scFv) and a humanized antibody (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, New York; Houston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird *et al.*, 1988, Science 242:423-426). In one embodiment, the antigen binding domain of a CAR comprises an antibody fragment. In a further embodiment, the CAR comprises an antibody fragment that comprises an scFv.
- 10 [0047] As used herein, the term "binding domain" or "antibody molecule" refers to a protein, *e.g.*, an immunoglobulin chain or fragment thereof, comprising at least one immunoglobulin variable domain sequence. The term "binding domain" or "antibody molecule" encompasses antibodies and antibody fragments. In an embodiment, an antibody molecule is a multispecific antibody molecule, *e.g.*, it comprises a plurality of immunoglobulin variable domain sequences, 15 wherein a first immunoglobulin variable domain sequence of the plurality has binding specificity for a first epitope and a second immunoglobulin variable domain sequence of the plurality has binding specificity for a second epitope. In an embodiment, a multispecific antibody molecule is a bispecific antibody molecule. A bispecific antibody has specificity for no more than two antigens. A bispecific antibody molecule is characterized by a first immunoglobulin variable 20 domain sequence which has binding specificity for a first epitope and a second immunoglobulin variable domain sequence that has binding specificity for a second epitope. The portion of the CAR of the invention comprising an antibody or antibody fragment thereof may exist in a variety of forms where the antigen binding domain is expressed as part of a contiguous polypeptide chain including, for example, a single domain antibody fragment (sdAb), a single 25 chain antibody (scFv), a humanized antibody, or bispecific antibody (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, New York; Houston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird *et al.*, 1988, Science 242:423-426). In one aspect, the antigen binding domain of a CAR composition of the invention comprises an 30 antibody fragment. In a further aspect, the CAR comprises an antibody fragment that comprises an scFv.

[0048] The term "antibody heavy chain," refers to the larger of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations, and which normally determines the class to which the antibody belongs.

[0049] The term "antibody light chain," refers to the smaller of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations. Kappa (κ) and lambda (λ) light chains refer to the two major antibody light chain isotypes. The term "complementarity determining region" or "CDR," as used herein, refers to the sequences of amino acids within antibody variable regions which confer antigen specificity and binding affinity. For example, in general, there are three CDRs in each heavy chain variable region (*e.g.*, HCDR1, HCDR2, and HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, and LCDR3). The precise amino acid sequence boundaries of a given CDR can be determined using any of a number of well-known schemes, including those described by Kabat *et al.* (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD ("Kabat" numbering scheme), Al-Lazikani *et al.*, (1997) JMB 273,927-948 ("Chothia" numbering scheme), or a combination thereof. Under the Kabat numbering scheme, in some embodiments, the CDR amino acid residues in the heavy chain variable domain (VH) are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the light chain variable domain (VL) are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under the Chothia numbering scheme, in some embodiments, the CDR amino acids in the VH are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the VL are numbered 26-32 (LCDR1), 50-52 (LCDR2), and 91-96 (LCDR3). In a combined Kabat and Chothia numbering scheme, in some embodiments, the CDRs correspond to the amino acid residues that are part of a Kabat CDR, a Chothia CDR, or both. For instance, in some embodiments, the CDRs correspond to amino acid residues 26-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3) in a VH, *e.g.*, a mammalian VH, *e.g.*, a human VH; and amino acid residues 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3) in a VL, *e.g.*, a mammalian VL, *e.g.*, a human VL. The term "recombinant antibody" refers to an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage or yeast expression system. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using recombinant DNA or amino acid sequence technology which is available and well known in the art.

[0050] The term "antigen" or "Ag" refers to a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen.

5 [0051] Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an "antigen" as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily
10 apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to encode polypeptides that elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a "gene" at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological
15 sample, or might be macromolecule besides a polypeptide. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a fluid with other biological components.

[0052] The term "autologous" refers to any material derived from the same individual to whom it is later to be re-introduced into the individual.

20 [0053] The term "allogeneic" refers to any material derived from a different animal of the same species as the individual to whom the material is introduced. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical. In some aspects, allogeneic material from individuals of the same species may be sufficiently unlike genetically to interact antigenically. The term "xenogeneic" refers to any material derived from
25 an animal of a different species.

[0054] The term "cancer" refers to a disease characterized by the uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. A cancer or disease can be one or more of the following: acoustic neuroma, adenocarcinoma, adrenal gland cancer, anal cancer, angiosarcoma (*e.g.*,
30 lymphangiosarcoma, lymphangioendotheliosarcoma, hemangiosarcoma), benign monoclonal gammopathy, biliary cancer (*e.g.*, cholangiocarcinoma), bladder cancer, breast cancer (*e.g.*, adenocarcinoma of the breast, papillary carcinoma of the breast, mammary cancer, medullary carcinoma of the breast), brain cancer (*e.g.*, meningioma; glioma, *e.g.*, astrocytoma,

Attorney Docket No.: VSM-064WO

oligodendroglioma; medulloblastoma), bronchus cancer, cervical cancer (*e.g.*, cervical adenocarcinoma), choriocarcinoma, chordoma, craniopharyngioma, colorectal cancer (*e.g.*, colon cancer, rectal cancer, colorectal adenocarcinoma), epithelial carcinoma, ependymoma, endotheliosarcoma (*e.g.*, Kaposi's sarcoma, multiple idiopathic hemorrhagic sarcoma),
 5 endometrial cancer, esophageal cancer (*e.g.*, adenocarcinoma of the esophagus, Barrett's adenocarcinoma), Ewing sarcoma, familial hypereosinophilia, gastric cancer (*e.g.*, stomach adenocarcinoma), gastrointestinal stromal tumor (GIST), head and neck cancer (*e.g.*, head and neck squamous cell carcinoma, oral cancer (*e.g.*, oral squamous cell carcinoma (OSCC))), heavy chain disease (*e.g.*, alpha chain disease, gamma chain disease, mu chain disease),
 10 hemangioblastoma, inflammatory myofibroblastic tumors, immunocytic amyloidosis, kidney cancer (*e.g.*, nephroblastoma a.k.a. Wilms' tumor, renal cell carcinoma), liver cancer (*e.g.*, hepatocellular cancer (HCC), malignant hepatoma), lung cancer (*e.g.*, bronchogenic carcinoma, small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung), leukemia (*e.g.*, acute lymphocytic leukemia (ALL), which includes B-lineage ALL and T-
 15 lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocyte leukemia (PLL), hairy cell leukemia (HCL) and Waldenstrom's macroglobulinemia (WM); peripheral T cell lymphomas (PTCL), adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGL), Hodgkin's disease and Reed-Stenberg disease; acute myelocytic leukemia (AML), chronic myelocytic leukemia (CML), chronic lymphocytic
 20 leukemia (CLL)), lymphoma (*e.g.*, Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), follicular lymphoma, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL)), leiomyosarcoma (LMS), mastocytosis (*e.g.*, systemic mastocytosis), multiple myeloma (MM), myelodysplastic syndrome (MDS), mesothelioma, myeloproliferative disorder (MPD) (*e.g.*, polycythemia Vera (PV), essential thrombocytosis (ET), agnogenic myeloid metaplasia (AMM)
 25 also known as myelofibrosis (MF), chronic idiopathic myelofibrosis, chronic myelocytic leukemia (CML), chronic neutrophilic leukemia (CNL), hypereosinophilic syndrome (HES)), neuroblastoma, neurofibroma (*e.g.*, neurofibromatosis (NF) type 1 or type 2, schwannomatosis), neuroendocrine cancer (*e.g.*, gastroenteropancreatic neuroendocrine tumor (GEP-NET), carcinoid tumor), osteosarcoma, ovarian cancer (*e.g.*, cystadenocarcinoma, ovarian embryonal
 30 carcinoma, ovarian adenocarcinoma), Paget's disease of the vulva, Paget's disease of the penis, papillary adenocarcinoma, pancreatic cancer (*e.g.*, pancreatic adenocarcinoma, intraductal papillary mucinous neoplasm (IPMN)), pinealoma, primitive neuroectodermal tumor (PNET), prostate cancer (*e.g.*, prostate adenocarcinoma), rhabdomyosarcoma, retinoblastoma, salivary gland cancer, skin cancer (*e.g.*, squamous cell carcinoma (SCC), keratoacanthoma (KA),

Attorney Docket No.: VSM-064WO

melanoma, basal cell carcinoma (BCC)), small bowel cancer (*e.g.*, appendix cancer), soft tissue sarcoma (*e.g.*, malignant fibrous histiocytoma (MFH), liposarcoma, malignant peripheral nerve sheath tumor (MPNST), chondrosarcoma, fibrosarcoma, myxosarcoma), sebaceous gland carcinoma, sweat gland carcinoma, synovial sarcoma, testicular cancer (*e.g.*, seminoma, testicular embryonal carcinoma), thyroid cancer (*e.g.*, papillary carcinoma of the thyroid, papillary thyroid carcinoma (PTC), medullary thyroid cancer), and Waldenstrom's macroglobulinemia.

[0055] In some embodiments, the cancer or disease is a blood disorder or a hematologic malignancy, including, but not limited to, myeloid disorder, lymphoid disorder, leukemia, lymphoma, myelodysplastic syndrome (MDS), myeloproliferative disease (MPD), mast cell disorder, and myeloma (*e.g.*, multiple myeloma), among others. In some embodiments, the blood disorder or the hematologic malignancy includes, but is not limited to, acute lymphoblastic leukemia (ALL), T-cell ALL (T-ALL), B-cell ALL (B-ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), blast phase CML, small lymphocytic lymphoma (SLL), CLL/SLL, blast phase CLL, Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), B-cell NHL, T-cell NHL, indolent NHL (iNHL), diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), aggressive B-cell NHL, B-cell lymphoma (BCL), Richter's syndrome (RS), T-cell lymphoma (TCL), peripheral T-cell lymphoma (PTCL), cutaneous T-cell lymphoma (CTCL), transformed mycosis fungoides, Sezary syndrome, anaplastic large-cell lymphoma (ALCL), follicular lymphoma (FL), Waldenstrom macroglobulinemia (WM), lymphoplasmacytic lymphoma, Burkitt lymphoma, multiple myeloma (MM), amyloidosis, MPD, essential thrombocytosis (ET), myelofibrosis (MF), polycythemia vera (PV), chronic myelomonocytic leukemia (CMML), myelodysplastic syndrome (MDS), angioimmunoblastic lymphoma, high-risk MDS, and low-risk MDS. In certain embodiments, the cancer is CLL. In some embodiments, the hematologic malignancy is relapsed. In some embodiments, the hematologic malignancy is refractory. In some embodiments, the cancer or disease is in a pediatric patient (including an infantile patient). In some embodiments, the cancer or disease is in an adult patient. Additional embodiments of a cancer or disease being treated or prevented by methods, compositions, or kits provided herein are described herein elsewhere.

[0056] In some embodiments, the cancer is a solid tumor, including, but not limited to, adenocarcinoma, bladder cancer, breast cancer, brain cancer, cervical cancer, colorectal cancer, endometrial cancer, esophageal cancer, gastric cancer, head and neck cancer, kidney cancer, liver cancer, lung cancer, neuroendocrine cancer, osteosarcoma, ovarian cancer, pancreatic

Attorney Docket No.: VSM-064WO

cancer, prostate cancer, retinoblastoma, salivary gland cancer, skin cancer, soft tissue sarcoma, testicular cancer, thyroid cancer, and a cancer as described above. In some embodiments, the malignancy is relapsed. In some embodiments, the malignancy is refractory. In some embodiments, the cancer or disease is in a pediatric patient (including an infantile patient). In some embodiments, the cancer or disease is in an adult patient. Additional embodiments of a cancer or disease being treated or prevented by methods, compositions, or kits provided herein are described herein elsewhere.

[0057] The terms "tumor" and "cancer" are used interchangeably herein, *e.g.*, both terms encompass solid and liquid, *e.g.*, diffuse or circulating, tumors. As used herein, the term "cancer" or "tumor" includes premalignant, as well as malignant cancers and tumors.

[0058] The phrase "disease associated with expression of a tumor antigen as described herein" includes, but is not limited to, a disease associated with expression of a tumor antigen as described herein or condition associated with cells which express a tumor antigen as described herein including, *e.g.*, proliferative diseases such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells which express a tumor antigen as described herein. In one embodiment, a cancer associated with expression of a tumor antigen as described herein is a hematological cancer. In one embodiment, a cancer associated with expression of a tumor antigen as described herein is a solid cancer. Further diseases associated with expression of a tumor antigen as described herein include, but not limited to, *e.g.*, atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of a tumor antigen as described herein. Non-cancer related indications associated with expression of a tumor antigen as described herein include, but are not limited to, *e.g.*, autoimmune disease, (*e.g.*, lupus), inflammatory disorders (allergy and asthma) and transplantation. In some embodiments, the tumor antigen-expressing cells express, or at any time expressed, mRNA encoding the tumor antigen. In an embodiment, the tumor antigen-expressing cells produce the tumor antigen protein (*e.g.*, wild-type or mutant), and the tumor antigen protein may be present at normal levels or reduced levels. In an embodiment, the tumor antigen-expressing cells produced detectable levels of a tumor antigen protein at one point, and subsequently produced substantially no detectable tumor antigen protein.

[0059] The phrase "disease associated with expression of CD19" includes, but is not limited to, a disease associated with expression of CD19 or condition associated with cells which express CD19 including, *e.g.*, proliferative diseases such as a cancer or malignancy or a

Attorney Docket No.: VSM-064WO

precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells which express CD19. In one aspect, a cancer associated with expression of CD19 is a hematological cancer. In one aspect, the hematological cancer is a leukemia or a lymphoma. In one aspect, a cancer associated with

5 expression of CD19 includes cancers and malignancies including, but not limited to, *e.g.*, one or more acute leukemias including but not limited to, *e.g.*, acute myeloid leukemia (AML), B-cell acute Lymphoid Leukemia (BALL), T-cell acute Lymphoid Leukemia (TALL), acute lymphoid leukemia (ALL); one or more chronic leukemias including but not limited to, *e.g.*, chronic myelogenous leukemia (CML), Chronic Lymphoid Leukemia (CLL). Additional cancers or

10 hematologic conditions associated with expression of CD19 comprise, but are not limited to, *e.g.*, B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, Follicular lymphoma, Hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, mantle cell lymphoma (MCL), Marginal zone lymphoma, multiple myeloma,

15 myelodysplasia and myelodysplastic syndrome, non-Hodgkin lymphoma, Hodgkin lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, myeloproliferative neoplasm; a histiocytic disorder (*e.g.*, a mast cell disorder or a blastic plasmacytoid dendritic cell neoplasm); a mast cell disorder, *e.g.*, systemic mastocytosis or mast cell leukemia; B-cell prolymphocytic leukemia, plasma cell myeloma, and

20 "preleukemia" which are a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells, and the like. Further diseases associated with expression of CD19 expression include, but not limited to, *e.g.*, atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of CD19. In some embodiments, the cancer to be treated is PTCL. In some

25 embodiments, the cancer to be treated is CTCL. In some embodiments, the cancer to be treated is MCL. In some embodiments, the subject to be treated is treatment naïve. In some embodiments, the subject to be treated has undergone previous treatment.

[0060] Non-cancer related indications associated with expression of CD19 include, but are not limited to, *e.g.*, autoimmune disease, (*e.g.*, lupus), inflammatory disorders (allergy and

30 asthma) and transplantation. In some embodiments, the tumor antigen- expressing cells express, or at any time expressed, mRNA encoding the tumor antigen. In an embodiment, the tumor antigen-expressing cells produce the tumor antigen protein (*e.g.*, wild-type or mutant), and the tumor antigen protein may be present at normal levels or reduced levels. In an embodiment, the tumor antigen -expressing cells produced detectable levels of a tumor antigen protein at one

point, and subsequently produced substantially no detectable tumor antigen protein. In other embodiments, the disease is a CD19-negative cancer, *e.g.*, a CD19-negative relapsed cancer. In some embodiments, the tumor antigen (*e.g.*, CD19)-expressing cell expresses, or at any time expressed, mRNA encoding the tumor antigen. In an embodiment, the tumor antigen (*e.g.*,
5 CD19)-expressing cell produces the tumor antigen protein (*e.g.*, wild-type or mutant), and the tumor antigen protein may be present at normal levels or reduced levels. In an embodiment, the tumor antigen (*e.g.*, CD19)-expressing cell produced detectable levels of a tumor antigen protein at one point, and subsequently produced substantially no detectable tumor antigen protein.

[0061] The phrase "disease associated with expression of a B-cell antigen" includes, but is
10 not limited to, a disease associated with expression of one or more of CD19, CD20, CD22 or ROR1, or a condition associated with cells which express, or at any time expressed, one or more of CD19, CD20, CD22 or ROR1, including, *e.g.*, proliferative diseases such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells which express one or
15 more of CD19, CD20, CD22 or ROR1. For the avoidance of doubt, a disease associated with expression of the B-cell antigen may include a condition associated with cells which do not presently express the B-cell antigen, *e.g.*, because the antigen expression has been downregulated, *e.g.*, due to treatment with a molecule targeting the B-cell antigen, *e.g.*, a B-cell targeting CAR, but which at one time expressed the antigen. The phrase "disease associated with
20 expression of a B-cell antigen" includes a disease associated with expression of CD19, as described herein. In embodiments, the CAR-expressing cells are used to treat a disease associated with a B-cell antigen. In embodiments, a CAR produced by a method herein comprises an antigen binding domain that targets a B-cell antigen.

[0062] The term "conservative sequence modifications" refers to amino acid modifications
25 that do not significantly affect or alter the binding characteristics of the antibody or antibody fragment containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody or antibody fragment of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are
30 ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine,

Attorney Docket No.: VSM-064WO

asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within a CAR described herein can
5 be replaced with other amino acid residues from the same side chain family and the altered CAR can be tested using the functional assays described herein.

[0063] The term "stimulation," refers to a primary response induced by binding of a stimulatory molecule (e.g., a TCR/CD3 complex or CAR) with its cognate ligand (or tumor antigen in the case of a CAR) thereby mediating a signal transduction event, such as, but not
10 limited to, signal transduction via the TCR/CD3 complex or signal transduction via the appropriate NK receptor or signaling domains of the CAR. Stimulation can mediate altered expression of certain molecules.

[0064] The term "stimulatory molecule," refers to a molecule expressed by an immune cell (e.g., T cell, NK cell, B cell) that provides the cytoplasmic signaling sequence(s) that regulate
15 activation of the immune cell in a stimulatory way for at least some aspect of the immune cell signaling pathway. In one aspect, the signal is a primary signal that is initiated by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, and which leads to mediation of a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A primary cytoplasmic signaling sequence (also referred to as a
20 "primary signaling domain") that acts in a stimulatory manner may contain a signaling motif which is known as immunoreceptor tyrosine-based activation motif or ITAM. Examples of an ITAM containing cytoplasmic signaling sequence that is of particular use in the invention includes, but is not limited to, those derived from CD3 zeta, common FcR gamma (FCER1G), Fc gamma R1a, FcR beta (Fc Epsilon Rib), CD3 gamma, CD3 delta, CD3 epsilon, CD79a,
25 CD79b, DAP10, and DAP12. In a specific CAR of the invention, the intracellular signaling domain in any one or more CARS of the invention comprises an intracellular signaling sequence, e.g., a primary signaling sequence of CD3-zeta.

[0065] IC₅₀ refers to a measure of the effectiveness of a therapeutic agent in inhibiting cancer cells by 50%.

30 [0066] The term "antigen presenting cell" or "APC" refers to an immune system cell such as an accessory cell (e.g., a B-cell, a dendritic cell, and the like) that displays a foreign antigen complexed with major histocompatibility complexes (MHC's) on its surface. T-cells may

recognize these complexes using their T-cell receptors (TCRs). APCs process antigens and present them to T-cells.

[0067] An "intracellular signaling domain," as the term is used herein, refers to an intracellular portion of a molecule. The intracellular signaling domain can generate a signal that promotes an immune effector function of the CAR containing cell, *e.g.*, a CAR-T cell. Examples of immune effector function, *e.g.*, in a CAR-T cell, include cytolytic activity and helper activity, including the secretion of cytokines. In embodiments, the intracellular signaling domain is the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function. While the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal. In an embodiment, the intracellular signaling domain can comprise a primary intracellular signaling domain. Exemplary primary intracellular signaling domains include those derived from the molecules responsible for primary stimulation, or antigen dependent stimulation. In an embodiment, the intracellular signaling domain can comprise a costimulatory intracellular domain. Exemplary costimulatory intracellular signaling domains include those derived from molecules responsible for costimulatory signals, or antigen independent stimulation. For example, in the case of a CAR-T, a primary intracellular signaling domain can comprise a cytoplasmic sequence of a T cell receptor, and a costimulatory intracellular signaling domain can comprise cytoplasmic sequence from co-receptor or costimulatory molecule. A primary intracellular signaling domain can comprise a signaling motif which is known as an immunoreceptor tyrosine-based activation motif or ITAM. Examples of ITAM containing primary cytoplasmic signaling sequences include, but are not limited to, those derived from CD3 zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, CD278 ("ICOS"), FcRI, and CD66d, CD32, DAP10, and DAP12.

[0068] The term "zeta" or alternatively "zeta chain", "CD3-zeta" or "TCR-zeta" is defined as the protein provided as GenBank Acc. No. BAG36664.1, or the equivalent residues from a non-human species, *e.g.*, mouse, rodent, monkey, ape and the like, and a "zeta stimulatory domain" or alternatively a "CD3-zeta stimulatory domain" or a "TCR-zeta stimulatory domain" is defined as the amino acid residues from the cytoplasmic domain of the zeta chain that are sufficient to functionally transmit an initial signal necessary for T cell activation. In one aspect the

Attorney Docket No.: VSM-064WO

cytoplasmic domain of zeta comprises residues 52 through 164 of GenBank Acc. No.

BAG36664.1 or the equivalent residues from a non-human species, *e.g.*, mouse, rodent, monkey, ape and the like, that are functional orthologs thereof.

[0069] The term "costimulatory molecule" refers to the cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules are cell surface molecules other than antigen receptors or their ligands that are required for an efficient immune response. Costimulatory molecules include, but are not limited to MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signalling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD 19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds CD83.

[0070] A costimulatory intracellular signaling domain refers to an intracellular portion of a costimulatory molecule. The intracellular signaling domain can comprise the entire intracellular portion, or the entire native intracellular signaling domain, of the molecule from which it is derived, or a functional fragment thereof.

[0071] The intracellular signaling domain can comprise the entire intracellular portion, or the entire native intracellular signaling domain, of the molecule from which it is derived, or a functional fragment thereof.

[0072] The term "4-1BB" refers to a member of the TNFR superfamily with an amino acid sequence provided as GenBank Acc. No. AAA62478.2, or the equivalent residues from a non-human species, *e.g.*, mouse, rodent, monkey, ape and the like; and a "4-1BB costimulatory domain" is defined as amino acid residues 214-255 of GenBank Acc. No. AAA62478.2, or the equivalent residues from a non-human species, *e.g.*, mouse, rodent, monkey, ape and the like.

[0073] "Immune effector cell," as used herein, refers to a cell that is involved in an immune response, *e.g.*, in the promotion of an immune effector response. Examples of immune effector cells include T cells, *e.g.*, alpha/beta T cells and gamma/delta T cells, B cells, natural killer (NK) cells, natural killer T (NKT) cells, mast cells, and myeloid-derived phagocytes.

5 [0074] "Immune effector function or immune effector response," as is used herein, refers to a function or response, *e.g.*, of an immune effector cell, that enhances or promotes an immune attack of a target cell. *E.g.*, an immune effector function or response refers a property of a T or NK cell that promotes killing or the inhibition of growth or proliferation, of a target cell. In the case of a T cell, primary stimulation and co-stimulation are examples of immune effector
10 function or response. The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity.

[0075] The term "encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, 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
15 sequence of nucleotides (*e.g.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein 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 is usually provided in sequence listings, and the non-coding
20 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.

[0076] Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or a RNA
25 may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

[0077] The term "expression" refers to the transcription and/or translation of a particular nucleotide sequence driven by a promoter. The term "transfer vector" refers to a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated
30 nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "transfer vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to further include non-plasmid

and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, a polylysine compound, liposome, and the like. Examples of viral transfer vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like. The term "expression vector" refers to a vector
5 comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, including cosmid, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses,
10 retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

[0078] The term "*in vivo*" refers to an event that takes place in a subject's body.

[0079] The term "*in vitro*" refers to an event that takes places outside of a subject's body. *In vitro* assays encompass cell-based assays in which cells alive or dead are utilized and may also
15 encompass a cell-free or biochemical assay in which no intact cells are utilized.

[0080] The term "*ex vivo*" refers to an event which involves treating or performing a procedure on a cell, tissue and/or organ which has been removed from a subject's body. When appropriate, the cell, tissue and/or organ may be returned to a subject's body in a method of surgery or treatment.

20 [0081] The term "lentivirus" refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses.

25 [0082] The term "lentiviral vector" refers to a vector derived from at least a portion of a lentivirus genome, including especially a self-inactivating lentiviral vector as provided in Milone *et al.*, Mol. Ther. 17(8): 1453-1464 (2009). Other examples of lentivirus vectors that may be used in the clinic, include but are not limited to, *e.g.*, the LENTIVECTOR® gene delivery technology from Oxford BioMedica, the LENTIMAX™ vector system from Lentigen and the
30 like. Nonclinical types of lentiviral vectors are also available and would be known to one skilled in the art.

[0083] The term "homologous" or "identity" refers to the subunit sequence identity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit; *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous or identical at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (*e.g.*, 9 of 10), are matched or homologous, the two sequences are 90% homologous.

[0084] "Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies and antibody fragments thereof are human immunoglobulins (recipient antibody or antibody fragment) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, a humanized antibody/antibody fragment can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications can further refine and optimize antibody or antibody fragment performance. In general, the humanized antibody or antibody fragment thereof will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or a significant portion of the FR regions are those of a human immunoglobulin sequence. The humanized antibody or antibody fragment can also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, Nature, 321: 522-525, 1986; Reichmann *et al.*, Nature, 332: 323-329, 1988; Presta, Curr. Op. Struct. Biol., 2: 593-596, 1992.

[0085] "Fully human" refers to an immunoglobulin, such as an antibody or antibody fragment, where the whole molecule is of human origin or consists of an amino acid sequence identical to a human form of the antibody or immunoglobulin.

[0086] The term "isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell. In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

[0087] The term "parenteral" administration of an immunogenic composition includes, *e.g.*, subcutaneous (s.c), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, intratumoral, or infusion techniques. The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), or a combination of a DNA or RNA thereof, and polymers thereof in either single- or double-stranded form. The term "nucleic acid" includes a gene, cDNA or an mRNA. In one embodiment, the nucleic acid molecule is synthetic (*e.g.*, chemically synthesized) or recombinant. Unless specifically limited, the term encompasses nucleic acids containing analogues or derivatives of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzner *et al.*, Nucleic Acid Res. 19:5081 (1991); Ohtsuka *et al.*, J. Biol. Chem. 260:2605-2608 (1985); and Rossolini *et al.*, Mol. Cell. Probes 8:91-98 (1994)). The terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides,

modified polypeptides, derivatives, analogs, fusion proteins, among others. A polypeptide includes a natural peptide, a recombinant peptide, or a combination thereof.

5 [0088] The term "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

[0089] The term "promoter/regulatory sequence" refers to a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are
10 required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner. The term "constitutive" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

15 [0090] The term "inducible" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

[0091] The term "tissue-specific" promoter refers to a nucleotide sequence which, when
20 operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter. The terms "cancer associated antigen" or "tumor antigen" interchangeably refers to a molecule (typically protein, carbohydrate or lipid) that is preferentially expressed on the surface of a cancer cell, either entirely or as a fragment (*e.g.*, MHC/peptide), in comparison to a normal
25 cell, and which is useful for the preferential targeting of a pharmacological agent to the cancer cell. In some embodiments, a tumor antigen is a marker expressed by both normal cells and cancer cells, *e.g.*, a lineage marker, *e.g.*, CD19 on B cells. In certain aspects, the tumor antigens of the present invention are derived from, cancers including but not limited to primary or metastatic melanoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, non-Hodgkin
30 lymphoma, Hodgkin lymphoma, leukemias, uterine cancer, cervical cancer, bladder cancer, kidney cancer and adenocarcinomas such as breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, and the like. In some embodiments, a cancer-associated antigen is a cell surface molecule that is overexpressed in a cancer cell in comparison to a normal cell, for

instance, 1-fold over expression, 2-fold overexpression, 3 -fold overexpression or more in comparison to a normal cell. In some embodiments, a cancer-associated antigen is a cell surface molecule that is inappropriately synthesized in the cancer cell, for instance, a molecule that contains deletions, additions or mutations in comparison to the molecule expressed on a normal cell. In some embodiments, a cancer-associated antigen will be expressed exclusively on the cell surface of a cancer cell, entirely or as a fragment (*e.g.*, MHC/peptide), and not synthesized or expressed on the surface of a normal cell. In some embodiments, the CARs of the present invention include CARs comprising an antigen binding domain (*e.g.*, antibody or antibody fragment) that binds to a MHC presented peptide. Normally, peptides derived from endogenous proteins fill the pockets of Major histocompatibility complex (MHC) class I molecules, and are recognized by T cell receptors (TCRs) on CD8 + T lymphocytes. The MHC class I complexes are constitutively expressed by all nucleated cells. In cancer, virus-specific and/or tumor-specific peptide/MHC complexes represent a unique class of cell surface targets for immunotherapy. TCR-like antibodies targeting peptides derived from viral or tumor antigens in the context of human leukocyte antigen (HLA)-A1 or HLA-A2 have been described (see, *e.g.*, Sastry *et al.*, J Virol. 2011 85(5): 1935- 1942; Sergeeva *et al.*, Blood, 2011 117(16):4262-4272; Verma *et al.*, J Immunol 2010 184(4):2156-2165; Willemsen *et al.*, Gene Ther 2001 8(21) : 1601-1608 ; Dao *et al.*, Sci Transl Med 2013 5(176) : 176ra33 ; Tassev *et al.*, Cancer Gene Ther 2012 19(2):84-100). For example, TCR-like antibody can be identified from screening a library, such as a human scFv phage displayed library.

[0092] The term "flexible polypeptide linker" or "linker" as used in the context of an ScFv refers to a peptide linker that consists of amino acids such as glycine and/or serine residues used alone or in combination, to link variable heavy and variable light chain regions together. In one embodiment, the flexible polypeptide linker is a Gly/Ser linker and comprises the amino acid sequence (Gly-Gly-Gly-Ser)_n, where n is a positive integer equal to or greater than 1, or *e.g.*, n=1, n=2, n=3, n=4, n=5, n=6, n=7, n=8, n=9 and n=10.

[0093] The term "subject" is intended to include living organisms in which an immune response can be elicited (*e.g.*, mammals, human).

[0094] The term, a "substantially purified" cell refers to a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they

Attorney Docket No.: VSM-064WO

are naturally associated in their natural state. In some aspects, the cells are cultured *in vitro*. In other aspects, the cells are not cultured *in vitro*.

[0095] "Combination therapy", or "in combination with" refer to the use of more than one compound or agent to treat a particular disorder or condition. For example, a CAR therapy may be administered in combination with a second treatment method, *e.g.*, a second inhibitor or a chemotherapeutic. By "in combination with," it is not intended to imply that the other therapy and the CAR therapy must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of this disclosure. The CAR therapy can be administered concurrently with, prior to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, 12 weeks, or 16 weeks before), or subsequent to (*e.g.*, 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, 12 weeks, or 16 weeks after), the second treatment method. In general, each therapeutic agent will be administered at a dose and/or on a time schedule determined for that particular agent. The second treatment can be administered with the PI3K inhibitor in a single composition or separately in a different composition.

[0096] Ranges: 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. As another example, a range such as 95-99% identity, includes something with 95%, 96%, 97%, 98% or 99% identity, and includes subranges such as 96-99%, 96-98%, 96-97%, 97-99%, 97-98% and 98-99% identity. This applies regardless of the breadth of the range.

PI3K Inhibitors

[0097] PI3K inhibitors that can be used in the compositions and methods provided herein include, but are not limited to, those described in, *e.g.*, WO 09/088990, WO2011/008302, WO 2010/036380, WO 2010/006086, WO 09/114870, WO 05/113556, and US 2011/0046165, the entirety of each incorporated herein by reference. Additional PI3K inhibitors that can be used in

Attorney Docket No.: VSM-064WO

the compositions and methods provided herein include, but are not limited to, AMG-319, GSK 2126458 (2,4-Difluoro-N-{2-(methyloxy)-5-[4-(4-pyridazinyl)-6-quinolinyl]-3-pyridinyl}benzenesulfonamide), GSK 1059615 (5Z-[[4-(4-pyridinyl)-6-quinolinyl]methylene]-2,4-thiazolidinedione), GDC-0032 (4-[5,6-dihydro-2-[3-methyl-1-(1-methylethyl)-1H-1,2,4-triazol-5-yl]imidazo[1,2-d][1,4]benzoxazepin-9-yl]- α,α -dimethyl-1H-Pyrazole-1-acetamide), GDC-0980 ((S)-1-(4-((2-(2-aminopyrimidin-5-yl)-7-methyl-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methyl)piperazin-1-yl)-2-hydroxypropan-1-one), GDC-0941 (2-(1H-indazol-4-yl)-6-((4-(methylsulfonyl)piperazin-1-yl)methyl)-4-morpholinothieno[3,2-d]pyrimidine), XL147 (N-(3-(benzo[c][1,2,5]thiadiazol-5-ylamino)quinoxalin-2-yl)-4-methylbenzenesulfonamide), XL499, XL765 (SAR245409, N-[4-[[[3-[(3,5-dimethoxyphenyl)amino]-2-quinoxalinyl]amino]sulfonyl]phenyl]-3-methoxy-4-methyl-benzamide), PF-4691502 (2-amino-6-(6-methoxypyridin-3-yl)-4-methyl-8-[(1R,4R)-4-(2-hydroxyethoxy)cyclohexyl]-7H,8H-pyrido[2,3-d]pyrimidin-7-one), BKM 120 (buparlisib, 5-(2,6-dimorpholinopyrimidin-4-yl)-4-(trifluoromethyl)pyridin-2-amine), Idelalisib (CAL-101, GS 1101, (S)-2-(1-(9H-purin-6-ylamino)propyl)-5-fluoro-3-phenylquinazolin-4(3H)-one), CAL 263, SF1126 (3-[[2-[[5-[[amino(azaniumyl)methylidene]amino]-2-[[4-oxo-4-[4-(4-oxo-8-phenylchromen-2-yl)morpholin-4-ium-4-yl]oxybutanoyl]amino]pentanoyl]amino]acetyl]amino]-4-(1-carboxylatopropylamino)-4-oxobutanoate), PX-866 (sonolisib, [(3aR,6E,9S,9aR,10R,11aS)-6-[[bis(prop-2-enyl)amino]methylidene]-5-hydroxy-9-(methoxymethyl)-9a,11a-dimethyl-1,4,7-trioxo-2,3,3a,9,10,11-hexahydroindeno[4,5-h]isochromen-10-yl] acetate), BEZ235 (2-methyl-2-(4-(3-methyl-2-oxo-8-(quinolin-3-yl)-2,3-dihydroimidazo[4,5-c]quinolin-1-yl)phenyl)propanenitrile), GS9820 (CAL-120, (S)-2-(1-((9H-purin-6-yl)amino)ethyl)-6-fluoro-3-phenylquinazolin-4(3H)-one), BYL719 ((2S)-1,2-Pyrrolidinedicarboxamide, N1-[4-methyl-5-[2-(2,2,2-trifluoro-1,1-dimethylethyl)-4-pyridinyl]-2-thiazolyl]), RP6503, tenalisib (RP6530, (S)-2-(1-((7H-purin-6-yl)amino)propyl)-3-(3-fluorophenyl)-4H-chromen-4-one), TGR1202 (((S)-2-(1-(4-amino-3-(3-fluoro-4-isopropoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)ethyl)-6-fluoro-3-(3-fluorophenyl)-4H-chromen-4-one)), INK1117 (MLN-1117), BAY 80-6946 (2-amino-N-(7-methoxy-8-(3-morpholinopropoxy)-2,3-dihydroimidazo[1,2-c]quinazolin-5-yl)pyrimidine-5-carboxamide), IC87114 (2-((6-amino-9H-purin-9-yl)methyl)-5-methyl-3-otolylquinazolin-4(3H)-one), Palomid 529 (3-(4-methoxybenzyloxy)-8-(1-hydroxyethyl)-2-methoxy-6H-benzo[c]chromen-6-one), ZSTK474 (2-(difluoromethyl)-1-(4,6-dimorpholino-1,3,5-triazin-2-yl)-1H-benzo[d]imidazole), PWT33597, TG 100-115 (6,7-Bis(3-hydroxyphenyl)pteridine-2,4-diamine), GNE-477 (5-[7-methyl-4-(morpholin-4-yl)-6-[(4-methylsulfonylpiperazin-1-yl)methyl]thieno[3,2-d]pyrimidin-2-yl]pyrimidin-2-amine), CUDC-

Attorney Docket No.: VSM-064WO

907 (N-hydroxy-2-(((2-(6-methoxypyridin-3-yl)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methyl)(methyl)amino)pyrimidine-5-carboxamide), AEZS-136, BGT-226 (8-(6-methoxypyridin-3-yl)-3-methyl-1-(4-(piperazin-1-yl)-3-(trifluoromethyl)phenyl)-1H-imidazo[4,5-c]quinolin-2(3H)-one maleic acid), PF-05212384 (1-(4-(4-(dimethylamino)piperidine-1-carbonyl)phenyl)-3-(4-(4,6-dimorpholino-1,3,5-triazin-2-yl)phenyl)urea), LY3023414, PI-103 (3-[4-(4-morpholinyl)pyrido[3',2':4,5]furo[3,2-d]pyrimidin-2-yl]-phenol), INCB040093, CAL-130 ((S)-2-(1-((2-amino-9H-purin-6-yl)amino)ethyl)-5-methyl-3-(o-tolyl)quinazolin-4(3H)-one), LY294002 (2-Morpholin-4-yl-8-phenylchromen-4-one), wortmannin, AS252424 (5-[1-[5-(4-Fluoro-2-hydroxy-phenyl)-furan-2-yl]-meth-(Z)-ylidene]-thiazolidine-2,4-dione), IPI-549 (2-amino-N-[(1S)-1-[8-[2-(1-methylpyrazol-4-yl)ethynyl]-1-oxo-2-phenylisoquinolin-3-yl]ethyl]pyrazolo[1,5-a]pyrimidine-3-carboxamide), CZC24832 (5-(2-Amino-8-fluoro-[1,2,4]triazolo[1,5-a]pyridin-6-yl)-N-tert-butylpyridine-3-sulfonamide), duvelisib (INK-1197, IPI-145, Copiktra™, (S)-3-(1-((9H-purin-6-yl)amino)ethyl)-8-chloro-2-phenylisoquinolin-1(2H)-one), or AS-604850 (5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione).

[0098] In some embodiments, a PI3K inhibitor is a compound that inhibits one or more PI3K isoforms, *e.g.*, alpha, beta, delta, or gamma isoform. In some embodiments, a PI3K inhibitor is a compound that inhibits one or more PI3K isoforms with an IC₅₀ of less than about 1000 nM, less than about 900 nM, less than about 800 nM, less than about 700 nM, less than about 600 nM, less than about 500 nM, less than about 400 nM, less than about 300 nM, less than about 200 nM, less than about 100 nM, less than about 75 nM, less than about 50 nM, less than about 25 nM, less than about 20 nM, less than about 15 nM, less than about 10 nM, less than about 10 nM, less than about 5 nM, or less than about 1 nM.

[0099] In some embodiments, the PI3K inhibitor is a compound that inhibits alpha, beta, delta and gamma isoforms of PI3K. In another embodiment, the PI3K inhibitor is a compound that inhibits beta, delta, and gamma isoforms of PI3K. In another embodiment, the PI3K inhibitor is a compound that inhibits the delta and gamma isoforms of PI3K.

[0100] In some embodiments, the PI3K inhibitor is a PI3K isoform selective inhibitor. In some embodiments, the PI3K inhibitor is a PI3K alpha selective inhibitor. In another embodiment, the PI3K inhibitor is a PI3K beta selective inhibitor.

[0101] In some embodiments, the PI3K inhibitor is a PI3K gamma selective inhibitor. In some embodiments, the PI3K gamma selective inhibitor selectively inhibits PI3K gamma isoform over PI3K delta isoform. In some embodiments, the PI3K gamma selective inhibitor has

- a delta/gamma selectivity ratio of greater than 1, greater than about 5, greater than about 10, greater than about 50, greater than about 100, greater than about 200, greater than about 400, greater than about 600, greater than about 800, greater than about 1000, greater than about 1500, greater than about 2000, greater than about 5000, greater than about 10,000, or greater than about 20,000. In some embodiments, the PI3K gamma selective inhibitor has a delta/gamma selectivity ratio in the range of from greater than 1 to about 5, from about 5 to about 10, from about 10 to about 50, from about 50 to about 850, or greater than about 850. In some embodiments, the delta/gamma selectivity ratio is determined by dividing the inhibitor's IC₅₀ against PI3K delta isoform by the inhibitor's IC₅₀ against PI3K gamma isoform.
- 10 [0102] In some embodiments, the PI3K inhibitor is a PI3K gamma selective inhibitor. In some embodiments, the PI3K gamma selective inhibitor selectively inhibits PI3K gamma isoform over PI3K alpha isoform. In some embodiments, the PI3K gamma selective inhibitor has an alpha/gamma selectivity ratio of greater than 1, greater than about 5, greater than about 10, greater than about 50, greater than about 100, greater than about 200, greater than about 400, greater than about 600, greater than about 800, greater than about 1000, greater than about 1500, greater than about 2000, greater than about 5000, greater than about 10,000, or greater than about 20,000. In some embodiments, the PI3K gamma selective inhibitor has an alpha/gamma selectivity ratio in the range of from greater than 1 to about 5, from about 5 to about 10, from about 10 to about 50, from about 50 to about 850, or greater than about 850. In some
- 15 20 embodiments, the alpha/gamma selectivity ratio is determined by dividing the inhibitor's IC₅₀ against PI3K alpha isoform by the inhibitor's IC₅₀ against PI3K gamma isoform.
- [0103] In some embodiments, the PI3K inhibitor is a PI3K gamma selective inhibitor. In some embodiments, the PI3K gamma selective inhibitor selectively inhibits PI3K gamma isoform over PI3K beta isoform. In some embodiments, the PI3K gamma selective inhibitor has a beta/gamma selectivity ratio of greater than 1, greater than about 5, greater than about 10, greater than about 50, greater than about 100, greater than about 200, greater than about 400, greater than about 600, greater than about 800, greater than about 1000, greater than about 1500, greater than about 2000, greater than about 5000, greater than about 10,000, or greater than about 20,000. In some embodiments, the PI3K gamma selective inhibitor has a beta/gamma selectivity ratio in the range of from greater than 1 to about 5, from about 5 to about 10, from about 10 to about 50, from about 50 to about 850, or greater than about 850. In some
- 25 30 embodiments, the beta/gamma selectivity ratio is determined by dividing the inhibitor's IC₅₀ against PI3K beta isoform by the inhibitor's IC₅₀ against PI3K gamma isoform.

Attorney Docket No.: VSM-064WO

[0104] In some embodiments, the PI3K inhibitor is selective for both gamma and delta isoforms, as determined by IC_{50} from whole blood assays for both PI3K-delta and PI3K-gamma at clinically achievable plasma exposures. In some embodiments, the PI3K gamma and delta selective inhibitor selectively inhibits PI3K gamma and delta isoforms over PI3K beta isoform.

5 In some embodiments, the PI3K gamma and delta selective inhibitor has a beta/delta selectivity ratio of greater than 1, greater than about 5, greater than about 10, greater than about 50, greater than about 100, greater than about 200, greater than about 400, greater than about 600, greater than about 800, greater than about 1000, greater than about 1500, greater than about 2000, greater than about 5000, greater than about 10,000, or greater than about 20,000 and a

10 beta/gamma selectivity ratio of greater than 1, greater than about 5, greater than about 10, greater than about 50, greater than about 100, greater than about 200, greater than about 400, greater than about 600, greater than about 800, greater than about 1000, greater than about 1500, greater than about 2000, greater than about 5000, greater than about 10,000, or greater than about 20,000. In some embodiments, the PI3K gamma and delta selective inhibitor has a beta/delta

15 selectivity ratio in the range of from greater than 1 to about 5, from about 5 to about 10, from about 10 to about 50, from about 50 to about 850, or greater than about 850 and a beta/gamma selectivity ratio in the range of from greater than 1 to about 5, from about 5 to about 10, from about 10 to about 50, from about 50 to about 850, or greater than about 850. In some

embodiments, the beta/delta selectivity ratio is determined by dividing the inhibitor's IC_{50} against

20 PI3K beta isoform by the inhibitor's IC_{50} against PI3K delta isoform and the beta/gamma selectivity ratio is determined by dividing the inhibitor's IC_{50} against PI3K beta isoform by the inhibitor's IC_{50} against PI3K gamma isoform.

[0105] PI3K gamma inhibitors that can be used in the compositions and methods provided herein include, but are not limited to, IPI-549 (2-amino-N-[(1S)-1-[8-[2-(1-methylpyrazol-4-yl)ethynyl]-1-oxo-2-phenylisoquinolin-3-yl]ethyl]pyrazolo[1,5-a]pyrimidine-3-carboxamide),

25 CZC24832 (5-(2-Amino-8-fluoro-[1,2,4]triazolo[1,5-a]pyridin-6-yl)-N-tert-butylpyridine-3-sulfonamide), AS252424 (5-[1-[5-(4-Fluoro-2-hydroxy-phenyl)-furan-2-yl]-meth-(Z)-ylidene]-thiazolidine-2,4-dione), or AS-604850 (5-(2,2-Difluoro-benzo[1,3]dioxol-5-ylmethylene)-thiazolidine-2,4-dione. In some embodiments, the PI3K gamma inhibitor is IPI-549.

30 [0106] In some embodiments, the PI3K inhibitor is a PI3K delta/gamma dual inhibitor. In some embodiments, the PI3K delta/gamma dual inhibitor has an IC_{50} value against PI3K alpha that is at least 5X, 10X, 20X, 50X, 100X, 200X, 500X, or 1000X higher than its IC_{50} values against delta and gamma.

Attorney Docket No.: VSM-064WO

[0107] In some embodiments, the PI3K inhibitor may be administered daily, every other day, three times a week, twice a week, weekly, or bi-weekly. In certain embodiments, the PI3K inhibitor may be administered twice daily. The dosing schedule can include a "drug holiday," *e.g.*, the drug may be administered for two weeks on, one week off, or three weeks on, one week off, or four weeks on, one week off, etc., or continuously, without a drug holiday. The inhibitor may be administered orally, intravenously, intraperitoneally, topically, transdermally, intramuscularly, subcutaneously, intranasally, sublingually, or by any other route.

[0108] In some embodiments, a PI3K inhibitor described herein may be administered in multiple doses. Dosing may be about once, twice, three times, four times, five times, six times, or more than six times per day. Dosing may be about once a month, about once every two weeks, about once a week, or about once every other day. In some cases, continuous dosing is achieved and maintained as long as necessary.

[0109] In some embodiments, a PI3K inhibitor described herein may be administered before apheresis, after apheresis and before CAR-T administration, concurrently with CAR-T administration, after CAR-T administration, or a combination thereof.

[0110] Generally, doses of a PI3K inhibitor described herein, will range from about 0.0001 mg to about 100 mg per day, or about 0.001 mg to about 100 mg per day, or about 0.01 mg to about 100 mg per day, or about 0.1 mg to about 100 mg per day, or about 0.0001 mg to about 500 mg per day, or about 0.001 mg to about 500 mg per day, or about 0.01 mg to 1000 mg, or about 0.01 mg to about 500 mg per day, or about 0.1 mg to about 500 mg per day, or about 1 mg to 50 mg per day, or about 5 mg to 40 mg per day. For example, the dosage may be 1 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 35 mg, 50 mg, 55 mg, 60 mg, 65 mg, 70 mg, 75 mg, 80 mg, 85 mg, 90 mg, 95 mg, 100 mg, 150 mg, 200 mg, 250 mg, 300 mg, 350 mg, 400 mg, 450 mg, or 500 mg, including all values and ranges between these values. An exemplary dosage is about 10 to 30 mg per day.

[0111] In embodiments in which the PI3K inhibitor is duvelisib, the dosage may be in the range of about 1 mg to about 50 mg, or about 5 mg to 50 mg (*e.g.*, about 15 mg, about 30 mg, about 45 mg, or about 50 mg, including all values and ranges in between). The dosage may be administered once daily or twice daily. In some embodiments, the daily dose of duvelisib is 30mg to 50 mg (*e.g.*, about 30 mg, about 45 mg, or about 50 mg, including all values and ranges in between). In some, about 15 mg duvelisib is administered twice daily, for a total daily dose of 30 mg. In some, about 25 mg duvelisib is administered twice daily, for a total daily dose of 50 mg.

[0112] In some embodiments, administration of a dual PI3K δ/γ inhibitor, *e.g.*, duvelisib, alters cellular metabolism. In some embodiments, administration of a dual PI3K δ/γ inhibitor, *e.g.*, duvelisib, decreases glycolysis and increases oxidative phosphorylation. In some embodiments, administration of a dual PI3K δ/γ inhibitor increases mRNA expression levels of proteins involved in the cellular metabolism, *e.g.*, mitochondrial biogenesis, *e.g.*, PGC1 α . In some embodiments, administration of a dual PI3K δ/γ inhibitor decreases mRNA expression levels of proteins involved in the cellular metabolism, *e.g.*, GLUT1 and GLUT4 transporters.

[0113] In some embodiments, administration of a dual PI3K δ/γ inhibitor increases mitochondrial biogenesis. In some embodiments, administration of a dual PI3K δ/γ inhibitor increases T-cell mitochondrial mass and mitochondrial respiration. In some embodiments, an increase in mitochondrial area relative to total cell area is observed for dual PI3K δ/γ inhibitor cultured T-cells/CAR T-cells relative to control T-cells. In some embodiments, the increase in mitochondrial area relative to total cell area is by 1-fold, 2-fold, 3-fold, 4-fold, or 5-fold. In some embodiments, mitochondrial cross-sectional area as a percentage of total cell area increases by 1-fold, 2-fold, 3-fold, 4-fold, or 5-fold following administration of a dual PI3K δ/γ inhibitor. In certain embodiments, mitochondrial mass is determined using transmission electron microscopy. In certain embodiments, mitochondrial mass is determined using cardiolipin staining. In some embodiments, administration of a dual PI3K δ/γ inhibitor decreases AKT phosphorylation and phosphorylation of downstream effectors AMPK, and mTOR. In some embodiments, administration of a dual PI3K δ/γ inhibitor increases autophagy.

Sources of Immune effector cells

[0114] In one aspect, the disclosure features an immune effector cell (*e.g.*, T cell, NK cell) engineered to express a CAR, wherein the engineered immune effector cell exhibits an antitumor property. An exemplary antigen is a cancer associated antigen (*i.e.*, tumor antigen) described herein. In one aspect, a cell is transformed with the CAR and the CAR is expressed on the cell surface. In some embodiments, the cell (*e.g.*, T cell, NK cell) is transduced with a viral vector encoding a CAR. In some embodiments, the viral vector is a retroviral vector. In some embodiments, the viral vector is a lentiviral vector. In some such embodiments, the cell may stably express the CAR. In another embodiment, the cell (*e.g.*, T cell, NK cell) is transfected with a nucleic acid, *e.g.*, mRNA, cDNA, DNA, encoding a CAR. In some such embodiments, the cell may transiently express the CAR. Furthermore, the present invention provides CAR-expressing cell, *e.g.*, CAR-T compositions and their use in medicaments or methods for treating,

among other diseases, cancer or any malignancy or autoimmune diseases involving cells or tissues which express a tumor antigen as described herein. In one aspect, the CAR of the invention can be used to eradicate a normal cell that express a tumor antigen as described herein, thereby applicable for use as a cellular conditioning therapy prior to cell transplantation.

5 [0115] In embodiments, prior to expansion and genetic modification or other modification, a source of cells, *e.g.*, T cells or natural killer (NK) cells, can be obtained from a subject.

[0116] Examples of subjects include humans, monkeys, chimpanzees, dogs, cats, mice, rats, and transgenic species thereof. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue,
10 tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors.

[0117] In certain aspects of the present disclosure, immune effector cells, *e.g.*, T cells, can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. In one aspect, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes,
15 including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one aspect, the cells collected by apheresis may be washed to remove the plasma fraction and, optionally, to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack
20 magnesium or may lack many or all divalent cations.

[0118] Initial activation steps in the absence of calcium can lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi- automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics
25 Cell Saver 5) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media. In one aspect, T cells are isolated from peripheral blood lymphocytes by lysing
30 the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient or by counterflow centrifugal elutriation.

[0119] The methods described herein can include, *e.g.*, selection of a specific subpopulation of immune effector cells, *e.g.*, T cells, that are a T regulatory cell-depleted population, CD25+

depleted cells, using, *e.g.*, a negative selection technique, *e.g.*, described herein. In some embodiments, the population of T regulatory-depleted cells contains less than about 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1% of CD25+ cells.

- [0120] In one embodiment, T regulatory cells, *e.g.*, CD25+ T cells, are removed from the population using an anti-CD25 antibody, or fragment thereof, or a CD25-binding ligand, *e.g.* IL-2. In one embodiment, the anti-CD25 antibody, or fragment thereof, or CD25-binding ligand is conjugated to a substrate, *e.g.*, a bead, or is otherwise coated on a substrate, *e.g.*, a bead. In one embodiment, the anti-CD25 antibody, or fragment thereof, is conjugated to a substrate as described herein.
- 10 [0121] In some embodiments, the manufacturing methods comprise reducing the number of (*e.g.*, depleting) T_{REG} cells prior to manufacturing of the CAR-expressing cell. For example, manufacturing methods comprise contacting the sample, *e.g.*, the apheresis sample, with an anti-GITR antibody and/or an anti-CD25 antibody (or fragment thereof, or a CD25-binding ligand), to deplete T_{REG} cells prior to manufacturing of the CAR-expressing cell (*e.g.*, T cell, NK cell)
- 15 product.

- [0122] Without wishing to be bound by a particular theory, decreasing the level of negative regulators of immune cells (*e.g.*, decreasing the number of unwanted immune cells, *e.g.*, T_{REG} cells), in a subject prior to apheresis or during manufacturing of a CAR-expressing cell product can reduce the risk of a subject's relapse. In an embodiment, a subject is pre-treated with one or
- 20 more therapies that reduce T_{REG} cells prior to collection of cells for CAR-expressing cell product manufacturing, thereby reducing the risk of subject relapse to CAR-expressing cell treatment. In an embodiment, methods of decreasing T_{REG} cells include, but are not limited to, administration to the subject of one or more of cyclophosphamide, anti-GITR antibody, CD25- depletion, or a combination thereof. In an embodiment, methods of decreasing T_{REG} cells include, but are not
- 25 limited to, administration to the subject of one or more of cyclophosphamide, anti-GITR antibody, CD25 -depletion, mTOR inhibitor, or a combination thereof. Administration of one or more of cyclophosphamide, anti-GITR antibody, CD25- depletion, or a combination thereof, can occur before, during or after an infusion of the CAR- expressing cell product. Administration of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, mTOR inhibitor, or a
- 30 combination thereof, can occur before, during or after an infusion of the CAR-expressing cell product.

[0123] In some embodiments, the manufacturing methods comprise reducing the number of (*e.g.*, depleting) T_{REG} cells prior to manufacturing of the CAR-expressing cell. For example,

Attorney Docket No.: VSM-064WO

manufacturing methods comprise contacting the sample, *e.g.*, the apheresis sample, with an anti-GITR antibody and/or an anti-CD25 antibody (or fragment thereof, or a CD25-binding ligand), *e.g.*, to deplete T_{REG} cells prior to manufacturing of the CAR-expressing cell (*e.g.*, T cell, NK cell) product.

- 5 **[0124]** In an embodiment, a subject is pre-treated with cyclophosphamide prior to collection of cells for CAR-expressing cell product manufacturing, thereby reducing the risk of subject relapse to CAR-expressing cell treatment (*e.g.*, CTL019 treatment). In an embodiment, a subject is pre-treated with an anti-GITR antibody prior to collection of cells for CAR-expressing cell (*e.g.*, T cell or NK cell) product manufacturing, thereby reducing the risk of subject relapse to
- 10 CAR-expressing cell treatment. In an embodiment, the CAR-expressing cell (*e.g.*, T cell, NK cell) manufacturing process is modified to deplete T_{REG} cells prior to manufacturing of the CAR-expressing cell (*e.g.*, T cell, NK cell) product (*e.g.*, a CTL019 product). In an embodiment, CD25-depletion is used to deplete T_{REG} cells prior to manufacturing of the CAR-expressing cell (*e.g.*, T cell, NK cell) product (*e.g.*, a CTL019 product). In one embodiment, the population of
- 15 cells to be removed are neither the regulatory T cells or tumor cells, but cells that otherwise negatively affect the expansion and/or function of CAR-T cells, *e.g.* cells expressing CD14, CD11b, CD33, CD15, or other markers expressed by potentially immune suppressive cells. In one embodiment, such cells are envisioned to be removed concurrently with regulatory T cells and/or tumor cells, or following said depletion, or in another order.
- 20 **[0125]** Methods described herein can include a positive selection step. For example, T cells can be isolated by incubation with anti-CD3/anti-CD28 (*e.g.*, 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further
- 25 embodiment, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another embodiment, the time period is 10 to 24 hours, *e.g.*, 24 hours. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such as isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the
- 30 efficiency of capture of CD8⁺ T cells. Thus, by simply shortening or lengthening the time T cells are allowed to bind to the CD3/CD28 beads and/or by increasing or decreasing the ratio of beads to T cells (as described further herein), subpopulations of T cells can be preferentially selected for or against at culture initiation or at other time points during the process.

Additionally, by increasing or decreasing the ratio of anti-CD3 and/or anti-CD28 antibodies on the beads or other surface, subpopulations of T cells can be preferentially selected for or against at culture initiation or at other desired time points. In one embodiment, a T cell population can be selected that expresses one or more of IFN- α , TNF α , IL-17A, IL-2, IL-3, IL-4, GM-CSF, IL-10, IL-13, granzyme B, and perforin, or other appropriate molecules, *e.g.*, other cytokines. Methods for screening for cell expression can be determined, *e.g.*, by the methods described in PCT Publication No.: WO 2013/126712.

[0126] For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (*e.g.*, particles such as beads) can be varied. In certain aspects, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (*e.g.*, increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one aspect, a concentration of 10 billion cells/ml, 9 billion cells/ml, 8 billion cells/ml, 7 billion cells/ml, 6 billion cells/ml, or 5 billion cells/ml is used. In one aspect, a concentration of 1 billion cells/ml is used. In yet one aspect, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further aspects, concentrations of 125 or 150 million cells/ml can be used.

[0127] Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (*e.g.*, leukemic blood, tumor tissue, etc.). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8⁺ T cells that normally have weaker CD28 expression. In a related aspect, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (*e.g.*, particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4⁺ T cells express higher levels of CD28 and are more efficiently captured than CD8⁺ T cells in dilute concentrations. In one aspect, the concentration of cells used is 5×10^6 /ml. In other aspects, the concentration used can be from about 1×10^5 /ml to 1×10^6 /ml, and any integer value in between.

[0128] In other aspects, the cells may be incubated on a rotator for varying lengths of time at varying speeds at either 2-10 °C or at room temperature. In one embodiment, a plurality of the immune effector cells of the population do not express diacylglycerol kinase (DGK), *e.g.*, is DGK-deficient. In one embodiment, a plurality of the immune effector cells of the population do not

Attorney Docket No.: VSM-064WO

express Ikaros, *e.g.*, is Ikaros-deficient. In one embodiment, a plurality of the immune effector cells of the population do not express DGK and Ikaros, *e.g.*, is both DGK and Ikaros-deficient. T cells for stimulation can also be frozen after a washing step. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or culture media containing 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin and 7.5% DMSO, or 31.25% Plasmalyte-A, 31.25% Dextrose 5%, 0.45% NaCl, 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin, and 7.5% DMSO or other suitable cell freezing media containing for example, Hespan and PlasmaLyte A, the cells then are frozen to -80°C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at -20° C or in liquid nitrogen.

[0129] In certain aspects, cryopreserved cells are thawed and washed as described herein and allowed to rest for one hour at room temperature prior to activation using the methods of the present invention.

[0130] Also contemplated in the context of the invention is the collection of blood samples or apheresis product from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as T cells, isolated and frozen for later use in immune effector cell therapy for any number of diseases or conditions that would benefit from immune effector cell therapy, such as those described herein. In one aspect a blood sample or an apheresis is taken from a generally healthy subject. In certain aspects, a blood sample or an apheresis is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet developed a disease, and the cells of interest are isolated and frozen for later use. In certain aspects, the T cells may be expanded, frozen, and used at a later time. In certain aspects, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but prior to any treatments. In a further aspect, the cells are isolated from a blood sample or an apheresis from a subject prior to any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin,

Attorney Docket No.: VSM-064WO

azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, Cytosan, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation.

[0131] In a further aspect of the present invention, T cells are obtained from a patient directly following treatment that leaves the subject with functional T cells. In this regard, it has been observed that following certain cancer treatments, in particular treatments with drugs that damage the immune system, shortly after treatment during the period when patients would normally be recovering from the treatment, the quality of T cells obtained may be optimal or improved for their ability to expand *ex vivo*. Likewise, following *ex vivo* manipulation using the methods described herein, these cells may be in a preferred state for enhanced engraftment and *in vivo* expansion. Thus, it is contemplated within the context of the present invention to collect blood cells, including T cells, dendritic cells, or other cells of the hematopoietic lineage, during this recovery phase. Further, in certain aspects, mobilization (for example, mobilization with GM-CSF) and conditioning regimens can be used to create a condition in a subject wherein repopulation, recirculation, regeneration, and/or expansion of particular cell types is favored, especially during a defined window of time following therapy. Illustrative cell types include T cells, B cells, dendritic cells, and other cells of the immune system.

[0132] It is recognized that the methods of the application can utilize culture media conditions comprising 5% or less, for example 2%, human AB serum, and employ known culture media conditions and compositions, for example those described in Smith *et al.*, "Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement" Clinical & Translational Immunology (2015) 4, e31; doi: 10.1038/cti.2014.31. In one embodiment, the methods of the application can utilize culture media conditions comprising serum-free medium. In one embodiment, the serum free medium is OpTmizer CTS (LifeTech), Immunocult XF (Stemcell technologies), CellGro (CellGenix), TexMacs (Miltenyi), Stemline (Sigma), Xvivo15 (Lonza), PrimeXV (Irvine Scientific), or StemXVivo (RandD systems). The serum-free medium can be supplemented with a serum substitute such as ICSR (immune cell serum replacement) from LifeTech. The level of serum substitute (*e.g.*, ICSR) can be, *e.g.*, up to 5%, *e.g.*, about 1%, 2%, 3%, 4%, or 5%.

[0133] In an embodiment, the NK cells are obtained from the subject. In another embodiment, the NK cells are an NK cell line, *e.g.*, NK-92 cell line (Conkwest).

Allogeneic CAR

[0134] In embodiments described herein, the immune effector cell can be an allogeneic immune effector cell, *e.g.*, T cell or NK cell. For example, the cell can be an allogeneic T cell, *e.g.*, an allogeneic T cell lacking expression of a functional T cell receptor (TCR) and/or human leukocyte antigen (HLA), *e.g.*, HLA class I and/or HLA class II. A T cell lacking a functional TCR can be, *e.g.*, engineered such that it does not express any functional TCR on its surface, engineered such that it does not express one or more subunits that comprise a functional TCR (*e.g.*, engineered such that it does not express (or exhibits reduced expression) of TCR alpha, TCR beta, TCR gamma, TCR delta, TCR epsilon, and/or TCR zeta) or engineered such that it produces very little functional TCR on its surface. Alternatively, the T cell can express a substantially impaired TCR, *e.g.*, by expression of mutated or truncated forms of one or more of the subunits of the TCR. The term "substantially impaired TCR" means that this TCR will not elicit an adverse immune reaction in a host.

[0135] A T cell described herein can be, *e.g.*, engineered such that it does not express a functional HLA on its surface. For example, a T cell described herein, can be engineered such that cell surface expression HLA, *e.g.*, HLA class I and/or HLA class II, is downregulated. In some embodiments, downregulation of HLA may be accomplished by reducing or eliminating expression of beta-2 microglobulin (B2M).

[0136] In some embodiments, the T cell can lack a functional TCR and a functional HLA, *e.g.*, HLA class I and/or HLA class II.

[0137] Modified T cells that lack expression of a functional TCR and/or HLA can be obtained by any suitable means, including a knock out or knock down of one or more subunit of TCR or HLA. For example, the T cell can include a knock down of TCR and/or HLA using siRNA, shRNA, clustered regularly interspaced short palindromic repeats (CRISPR) transcription-activator like effector nuclease (TALEN), or zinc finger endonuclease (ZFN).

[0138] In some embodiments, the allogeneic cell can be a cell which does not express or expresses at low levels an inhibitory molecule, *e.g.* by any method described herein. For example, the cell can be a cell that does not express or expresses at low levels an inhibitory molecule, *e.g.*, that can decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (*e.g.*, CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (*e.g.*, TGFRbeta). Inhibition of an inhibitory molecule, *e.g.*, by inhibition at the DNA, RNA or

protein level, can optimize a CAR-expressing cell performance. In embodiments, an inhibitory nucleic acid, *e.g.*, an inhibitory nucleic acid, *e.g.*, a dsRNA, *e.g.*, an siRNA or shRNA, a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), *e.g.*, as described herein, can
 5 be used.

siRNA and shRNA to inhibit TCR or HLA in allogeneic CAR

[0139] In some embodiments, TCR expression and/or HLA expression can be inhibited using siRNA or shRNA that targets a nucleic acid encoding a TCR and/or HLA, and/or an inhibitory molecule described herein (*e.g.*, PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (*e.g.*,
 10 CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta), in a cell, *e.g.*, T cell.

[0140] Expression systems for siRNA and shRNAs, and exemplary shRNAs, are described, *e.g.*, in paragraphs 649 and 650 of International Application WO2015/142675, filed March 13,
 15 2015, which is incorporated by reference in its entirety.

CRISPR to inhibit TCR or HLA in allogeneic CAR

[0141] "CRISPR" or "CRISPR to TCR and/or HLA" or "CRISPR to inhibit TCR and/or HLA" as used herein refers to a set of clustered regularly interspaced short palindromic repeats, or a system comprising such a set of repeats. "Cas", as used herein, refers to a CRISPR-
 20 associated protein. A "CRISPR/Cas" system refers to a system derived from CRISPR and Cas which can be used to silence or mutate a TCR and/or HLA gene, and/or an inhibitory molecule described herein (*e.g.*, PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (*e.g.*, CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC
 25 class I, MHC class II, GAL9, adenosine, and TGFR beta), in a cell, *e.g.*, T cell.

[0142] The CRISPR/Cas system, and uses thereof, are described, *e.g.*, in paragraphs 651-658 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety. TALEN to inhibit TCR and/or HLA

[0143] "TALEN" or "TALEN to HLA and/or TCR" or "TALEN to inhibit HLA and/or TCR"
 30 refers to a transcription activator-like effector nuclease, an artificial nuclease which can be used to edit the HLA and/or TCR gene, and/or an inhibitory molecule described herein (*e.g.*, PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (*e.g.*, CEACAM-1, CEACAM-3 and/or CEACAM-

Attorney Docket No.: VSM-064WO

5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta), in a cell, *e.g.*, T cell. TALENs, and uses thereof, are described, *e.g.*, in paragraphs 659-665 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety.

Zinc finger nuclease to inhibit HLA and/or TCR in allogeneic CAR

[0144] "ZFN" or "Zinc Finger Nuclease", *e.g.* TALEN, an artificial nuclease which can be used to edit the HLA and/or TCR gene, and/or an inhibitory molecule described herein (*e.g.*, PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (*e.g.*, CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta), in a cell, *e.g.*, T cell. ZFNs, and uses thereof, are described, *e.g.*, in paragraphs 666-671 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety.

Chimeric Antigen Receptors (CAR)

[0145] In embodiments, the present invention provides immune effector cells (*e.g.*, T cells, NK cells) that are engineered to contain one or more CARs that direct the immune effector cells to cancer. This is achieved through an antigen binding domain on the CAR that is specific for a cancer associated antigen. There are two classes of cancer associated antigens (tumor antigens) that can be targeted by the CARs described herein: (1) cancer associated antigens that are expressed on the surface of cancer cells; and (2) cancer associated antigens that itself is intracellular, however, a fragment of such antigen (peptide) is presented on the surface of the cancer cells by MHC (major histocompatibility complex).

[0146] Accordingly, an immune effector cell, *e.g.*, obtained by a method described herein, can be engineered to contain a CAR that targets one of the following cancer associated antigens (tumor antigens): CD19, CD70, CD123, CD22, CD30, CD171, CS-1, CLL-1, CD33, EGFRvIII, GD2, GD3, BCMA, Tn Ag, PSMA, ROR1, FLT3, FAP, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, Mesothelin, IL-1 lRa, PSCA, VEGFR2, LewisY, CD24, PDGFR-beta, PRSS21, SSEA-4, CD20, Folate receptor alpha, ERBB2 (Her2/neu), MUC1, EGFR, NCAM, Prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, Fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, TSHR, GPRC5D, CXORF61, CD97, CD179a, ALK, Physialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20,

Attorney Docket No.: VSM-064WO

LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-Ia, legumain, HPV E6,E7, MAGE-A1, MAGE A1, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, prostein, survivin and telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG
 5 (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin B 1, MYCN, RhoC, TRP-2, CYP1B 1, BORIS, SART3, PAX5, OY-TES 1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, and mut hsp70-2.

Bispecific CARs

[0147] In an embodiment a multispecific antibody molecule is a bispecific antibody
 10 molecule. A bispecific antibody has specificity for no more than two antigens. A bispecific antibody molecule is characterized by a first immunoglobulin variable domain sequence which has binding specificity for a first epitope and a second immunoglobulin variable domain sequence that has binding specificity for a second epitope. In an embodiment the first and second epitopes are on the same antigen, *e.g.*, the same protein (or subunit of a multimeric protein). In
 15 an embodiment the first and second epitopes overlap. In an embodiment the first and second epitopes do not overlap. In an embodiment the first and second epitopes are on different antigens, *e.g.*, different proteins (or different subunits of a multimeric protein). In an embodiment a bispecific antibody molecule comprises a heavy chain variable domain sequence and a light chain variable domain sequence which have binding specificity for a first epitope and
 20 a heavy chain variable domain sequence and a light chain variable domain sequence which have binding specificity for a second epitope. In an embodiment a bispecific antibody molecule comprises a half antibody having binding specificity for a first epitope and a half antibody having binding specificity for a second epitope. In an embodiment a bispecific antibody molecule comprises a half antibody, or fragment thereof, having binding specificity for a first
 25 epitope and a half antibody, or fragment thereof, having binding specificity for a second epitope. In an embodiment a bispecific antibody molecule comprises an scFv, or fragment thereof, have binding specificity for a first epitope and an scFv, or fragment thereof, have binding specificity for a second epitope. In certain embodiments, the antibody molecule is a multi-specific (*e.g.*, a bispecific or a trispecific) antibody molecule. Protocols for generating bispecific or
 30 heterodimeric antibody molecules, and various configurations for bispecific antibody molecules, are described in, *e.g.*, paragraphs 455-458 of WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety.

[0148] In one aspect, the bispecific antibody molecule is characterized by a first immunoglobulin variable domain sequence, *e.g.*, an scFv, which has binding specificity for CD 19, *e.g.*, comprises an scFv as described herein, or comprises the light chain CDRs and/or heavy chain CDRs from an scFv described herein, and a second immunoglobulin variable domain sequence that has binding specificity for a second epitope on a different antigen.

Chimeric TCR

[0149] In one aspect, the antibodies and antibody fragments of the present invention (*e.g.*, CD 19 antibodies and fragments) can be grafted to one or more constant domain of a T cell receptor ("TCR") chain, for example, a TCR alpha or TCR beta chain, to create a chimeric TCR. Without being bound by theory, it is believed that chimeric TCRs will signal through the TCR complex upon antigen binding. For example, an scFv as disclosed herein, can be grafted to the constant domain, *e.g.*, at least a portion of the extracellular constant domain, the transmembrane domain and the cytoplasmic domain, of a TCR chain, for example, the TCR alpha chain and/or the TCR beta chain. As another example, an antibody fragment, for example a VL domain as described herein, can be grafted to the constant domain of a TCR alpha chain, and an antibody fragment, for example a VH domain as described herein, can be grafted to the constant domain of a TCR beta chain (or alternatively, a VL domain may be grafted to the constant domain of the TCR beta chain and a VH domain may be grafted to a TCR alpha chain). As another example, the CDRs of an antibody or antibody fragment may be grafted into a TCR alpha and/or beta chain to create a chimeric TCR. For example, the LCDRs disclosed herein may be grafted into the variable domain of a TCR alpha chain and the HCDRs disclosed herein may be grafted to the variable domain of a TCR beta chain, or vice versa. Such chimeric TCRs may be produced, *e.g.*, by methods known in the art (For example, Willemsen RA et al, Gene Therapy 2000; 7: 1369-1377; Zhang T et al, Cancer Gene Ther 2004; 11: 487-496; Aggen et al, Gene Ther. 2012 Apr;19(4):365-74).

Non-Antibody Scaffolds

[0150] In embodiments, the antigen binding domain comprises a non-antibody scaffold, *e.g.*, a fibronectin, ankyrin, domain antibody, lipocalin, small modular immuno-pharmaceutical, maxibody, Protein A, or affilin. The non-antibody scaffold has the ability to bind to target antigen on a cell. In embodiments, the antigen binding domain is a polypeptide or fragment thereof of a naturally occurring protein expressed on a cell. In some embodiments, the antigen binding domain comprises a non-antibody scaffold. A wide variety of non-antibody scaffolds can be employed so long as the resulting polypeptide includes at least one binding region which

specifically binds to the target antigen on a target cell. Non-antibody scaffolds include: fibronectin (Novartis, MA), ankyrin (Molecular Partners AG, Zurich, Switzerland), domain antibodies (Domantis, Ltd., Cambridge, MA, and Ablynx nv, Zwijnaarde, Belgium), lipocalin (Pieris Proteolab AG, Freising, Germany), small modular immuno-pharmaceuticals (Trubion Pharmaceuticals Inc., Seattle, WA), maxybodies (Avidia, Inc., Mountain View, CA), Protein A (Affibody AG, Sweden), and affilin (gamma- crystallin or ubiquitin) (Scil Proteins GmbH, Halle, Germany).

10 [0151] In an embodiment, the antigen binding domain comprises the extracellular domain, or a counter-ligand binding fragment thereof, of molecule that binds a counterligand on the surface of a target cell.

15 [0152] The immune effector cells can comprise a recombinant DNA construct comprising sequences encoding a CAR, wherein the CAR comprises an antigen binding domain (*e.g.*, antibody or antibody fragment, TCR or TCR fragment) that binds specifically to a tumor antigen, *e.g.*, an tumor antigen described herein, and an intracellular signaling domain. The intracellular signaling domain can comprise a costimulatory signaling domain and/or a primary signaling domain, *e.g.*, a zeta chain. As described elsewhere, the methods described herein can include transducing a cell, *e.g.*, from the population of T regulatory-depleted cells, with a nucleic acid encoding a CAR, *e.g.*, a CAR described herein.

20 [0153] In specific aspects, a CAR comprises an scFv domain, wherein the scFv may be preceded by an optional leader sequence, and followed by an optional hinge sequence, a transmembrane region, an intracellular signalling domain and a CD3 zeta sequence, *e.g.*, wherein the domains are contiguous with and in the same reading frame such that they form a single fusion protein.

25 [0154] In one aspect, an exemplary CAR constructs comprise an optional leader sequence (*e.g.*, a leader sequence described herein), an extracellular antigen binding domain (*e.g.*, an antigen binding domain described herein), a hinge (*e.g.*, a hinge region described herein), a transmembrane domain (*e.g.*, a transmembrane domain described herein), and an intracellular stimulatory domain (*e.g.*, an intracellular stimulatory domain described herein). In one aspect, an exemplary CAR construct comprises an optional leader sequence (*e.g.*, a leader sequence described herein), an extracellular antigen binding domain (*e.g.*, an antigen binding domain described herein), a hinge (*e.g.*, a hinge region described herein), a transmembrane domain (*e.g.*, a transmembrane domain described herein), an intracellular costimulatory signaling domain

30

(*e.g.*, a costimulatory signaling domain described herein) and/or an intracellular primary signaling domain (*e.g.*, a primary signaling domain described herein).

[0155] In one aspect, the immune effector cell comprises a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a CAR, wherein the nucleic acid molecule comprises a nucleic acid sequence encoding an antigen binding domain, wherein the sequence is contiguous with and in the same reading frame as the nucleic acid sequence encoding an intracellular signaling domain. An exemplary intracellular signaling domain that can be used in the CAR includes, but is not limited to, one or more intracellular signaling domains of, *e.g.*, CD3-zeta, CD28, CD27, 4- 1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4- 1BB, and the like. The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the nucleic acid molecule, by deriving the nucleic acid molecule from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the nucleic acid of interest can be produced synthetically, rather than cloned. Nucleic acids encoding a CAR can be introduced into the immune effector cells using, *e.g.*, a retroviral or lentiviral vector construct.

[0156] Nucleic acids encoding a CAR can also be introduced into the immune effector cell using, *e.g.*, an RNA construct that can be directly transfected into a cell. A method for generating mRNA for use in transfection involves *in vitro* transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR") (*e.g.*, a 3' and/or 5' UTR described herein), a 5' cap (*e.g.*, a 5' cap described herein) and/or Internal Ribosome Entry Site (IRES) (*e.g.*, an IRES described herein), the nucleic acid to be expressed, and a polyA tail, typically 50-2000 bases in length. RNA thus produced can efficiently transfect different kinds of cells. In one embodiment, the template includes sequences for the CAR. In an embodiment, an RNA CAR vector is transduced into a cell, *e.g.*, a T cell by electroporation.

Antigen binding domain

[0157] In one aspect, a plurality of the immune effector cells, *e.g.*, the population of T regulatory-depleted cells, include a nucleic acid encoding a CAR that comprises a target-specific binding element otherwise referred to as an antigen binding domain. The choice of binding element depends upon the type and number of ligands that define the surface of a target cell. For example, the antigen binding domain may be chosen to recognize a ligand that acts as a

cell surface marker on target cells associated with a particular disease state. Thus, examples of cell surface markers that may act as ligands for the antigen binding domain in a CAR described herein include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells.

5 **[0158]** In one aspect, the portion of the CAR comprising the antigen binding domain comprises an antigen binding domain that targets a tumor antigen, *e.g.*, a tumor antigen described herein. The antigen binding domain can be any domain that binds to the antigen including but not limited to a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a human antibody, a humanized antibody, and a functional fragment thereof, including
10 but not limited to a single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (V_HH) of camelid derived nanobody, and to an alternative scaffold known in the art to function as antigen binding domain, such as a recombinant fibronectin domain, a T cell receptor (TCR), or a fragment there of, *e.g.*, single chain TCR, and the like. In some instances, it is beneficial for the antigen binding domain to be
15 derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial for the antigen binding domain of the CAR to comprise human or humanized residues for the antigen binding domain of an antibody or antibody fragment.

[0159] In an embodiment, the antigen binding domain comprises an anti-CD19 antibody, or antigen-binding fragment thereof, *e.g.*, an scFv. The antigen binding domain may comprise any
20 anti-CD19 antibody or antigen-binding fragment thereof known in the art. For example and without limitation, the antigen binding domain comprises a sequence of a CD19 binder listed in **Table 1**, or may share 85%, 90%, 95%, 97%, 98%, 99% sequence identity with an amino acid sequence listed in Table 1.

Table 1. Exemplary sequences of CD19-binders

Description	Sequence
Anti-CD19 binding domain scFv1	EIVMTQSPATLSLSPGERATLSCRASQDISKYLNWYQQKPGQAPRLLIYHTSRLHSGIPARFSGSGSGDYTLTISSLQPEDFAVYFCQQGNTLPYTFGQGTKLEIKGGGGSGGGGSGGGGSQVQLQESGPGLVKPSLTSLTCTVSGVSLPDYGVSWIRQPPGKGLEWIGVIWGSETTYSSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCAKHYYYGGSYAMDYWGQGTLVTVSS (SEQ ID NO:1)

Anti-CD19 binding domain scFv2	EIVMTQSPATLSLSPGERATLSCRASQDISKYLNWYQQKPGQAPRLLIY HTSRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFG QGTKLEIKGGGGSGGGGSGGGGSQVQLQESGPGLVKPSETLSLTCTVS GVSLPDYGVSWIRQPPGKGLEWIGVIWGSETTYQSSSLKSRVTISKDNS KNQVSLKLSSVTAADTAVYYCAKHYYYGGSYAMDYWGQGTLVTVSS (SEQ ID NO:2)
Anti-CD19 binding domain scFv3	QVQLQESGPGLVKPSETLSLTCTVSGVSLPDYGVSWIRQPPGKGLEWIG VIWGSETTYSSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCAKH YYYGGSYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSEIVMTQSPAT LSLSPGERATLSCRASQDISKYLNWYQQKPGQAPRLLIYHTSRLHSGIPAR FSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFGQGTKLEIK (SEQ ID NO:3)
Anti-CD19 binding domain scFv4	QVQLQESGPGLVKPSETLSLTCTVSGVSLPDYGVSWIRQPPGKGLEWIG VIWGSETTYQSSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCAK HYYYGGSYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSEIVMTQSP ATLSLSPGERATLSCRASQDISKYLNWYQQKPGQAPRLLIYHTSRLHSG IPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFGQGTKLEIK (SEQ ID NO:4)
Anti-CD19 binding domain scFv5	EIVMTQSPATLSLSPGERATISCRASQDISKYLNWYQQKPGQAPRLLIY HTSRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTF GQGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLQESGPGLVKPSET LSLTCTVSGVSLPDYGVSWIRQPPGKGLEWIGVIWGSETTYSSSLKS RVTISKDNSKNQVSLKLSSVTAADTAVYYCAKHYYYGGSYAMDYWG QGTLVTVSS (SEQ ID NO:5)
Anti-CD19 binding domain scFv6	EIVMTQSPATLSLSPGERATLSCRASQDISKYLNWYQQKPGQAPRLLIY HTSRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTF GQGTKLEIKGGGGSGGGGSGGGGSGGGGSQVQLQESGPGLVKPSETL SLTCTVSGVSLPDYGVSWIRQPPGKGLEWIGVIWGSETTYQSSSLKSR VTISKDNSKNQVSLKLSSVTAADTAVYYCAKHYYYGGSYAMDYWG QGTLVTVSS (SEQ ID NO:6)
Anti-CD19 binding	QVQLQESGPGLVKPSETLSLTCTVSGVSLPDYGVSWIRQPPGKGLEWI GVIWGSETTYSSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCAK

domain scFv7	HYYYGGSYAMDYWGQGT LVT VSSGGGGSGGGGSGGGGSGGGGSGGGGSEIV MTQSPATLSLSPGERATLSCRASQDISKYL N WYQQKPGQAPRLLIYHTS RLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFGQGT KLEIK (SEQ ID NO:7)
Anti-CD19 binding domain scFv8	QVQLQESGPGLVKPSETLSLTCTVSGVSLPDYGVSWIRQPPGKGLEWI GVIWGSETTY YQSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCA KHYYYGGSYAMDYWGQGT LVT VSSGGGGSGGGGSGGGGSGGGGSGGGGSE IVMTQSPATLSLSPGERATLSCRASQDISKYL N WYQQKPGQAPRLLIY HTSRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFG QGTKLEIK (SEQ ID NO:8)
Anti-CD19 binding domain scFv9	EIVMTQSPATLSLSPGERATISCRASQDISKYL N WYQQKPGQAPRLLIY HTSRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTF GQGTKLEIKGGGGSGGGGSGGGGSGGGGSGGGGSGVQLQESGPGLVKPSETLSL TCTVSGVSLPDYGVSWIRQPPGKGLEWIGVIWGSETTYYNSSLKSRVT ISKDNSKNQVSLKLSSVTAADTAVYYCAKHYYYGGSYAMDYWGQGT LVT VSS (SEQ ID NO:9)
Anti-CD19 binding domain scFv10	QVQLQESGPGLVKPSETLSLTCTVSGVSLPDYGVSWIRQPPGKGLEWI GVIWGSETTYYNSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCA KHYYYGGSYAMDYWGQGT LVT VSSGGGGSGGGGSGGGGSGGGGSGGGGSEI VMTQSPATLSLSPGERATISCRASQDISKYL N WYQQKPGQAPRLLIYHT SRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFGQ GTKLEIK (SEQ ID NO:10)
Anti-CD19 binding domain scFv11	EIVMTQSPATLSLSPGERATISCRASQDISKYL N WYQQKPGQAPRLLI YHTSRLHSGIPARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYT FGQGTKLEIKGGGGSGGGGSGGGGSGGGGSGVQLQESGPGLVKPSETLSLTC TVSGVSLPDYGVSWIRQPPGKGLEWIGVIWGSETTYYNSSLKSRVTIS KDNSKNQVSLKLSSVTAADTAVYYCAKHYYYGGSYAMDYWGQGT L VT VSS (SEQ ID NO:11)

Anti-CD19 binding domain scFv12	QVQLQESGPGLVKPSETLSLTCTVSGVSLPDYGVSWIRQPPGKGLEWI GVIWGSETTYYNSSLKSRVTISKDNSKNQVSLKLSSVTAADTAVYYCA KHYYYGGSYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSEIVMTQS PATLSLSPGERATISCRASQDISKYLNWYQQKPGQAPRLLIYHTSRHSGI PARFSGSGSGTDYTLTISSLQPEDFAVYFCQQGNTLPYTFGQGTKLEIK (SEQ ID NO:12)
Anti-CD19 binding domain scFv13	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIY HTSRLHSGVPSRFSGSGSGTDYSITISNLEQEDIATYFCQQGNTLPYTFG GGTKLEITGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTCTVS GVSLPDYGVSWIRQPPRKGLEWLGVIWGSETTYYNNSALKSRLTIKDN SKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVSS (SEQ ID NO:13)
Anti-CD19 binding domain VH	QVQLLESGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEQI GQIYPGDGDTNYNGKFKGQATLTADKSSSTAYMQLSGLTSEDSAVYSC ARKTISSVVDIFYFDYWGQGTTVT (SEQ ID NO:14)
Anti-CD19 binding domain VL	ELVLTQSPKFMSTSVGDRVSVTCKASQNVGTNVAWYQQKPGQS PKPLIYSATYRNSGVPDRFTGSGSGTDFTLTITNVQSKDLADYFYFCQYN RYPYTSGGGGTKLEIK (SEQ ID NO:15)

[0160] Any CD19 CAR, *e.g.*, the CD19 antigen binding domain of any known CD19 CAR, can be used in accordance with the present disclosure. For example, LG-740; CD19 CAR described in WO2019084288; US Pat. No. 8,399,645; US Pat. No. 7,446,190; Xu *et al.*, Leuk 5 Lymphoma. 2013 54(2):255-260(2012); Cruz *et al.*, Blood 122(17):2965-2973 (2013); Brentjens *et al.*, Blood, 118(18):4817-4828 (2011); Kochenderfer *et al.*, Blood 116(20):4099-102 (2010); Kochenderfer *et al.*, Blood 122 (25):4129-39(2013); and 16th Annu Meet Am Soc Gen Cell Ther (ASGCT) (May 15- 18, Salt Lake City) 2013, Abst 10.

[0161] In one embodiment, the antigen binding domain comprises one, two three (*e.g.*, all 10 three) heavy chain CDRs, HCDR1, HCDR2 and HCDR3, from an antibody listed above, and/or one, two, three (*e.g.*, all three) light chain CDRs, LCDR1, LCDR2 and LCDR3, from an antibody listed above. In one embodiment, the antigen binding domain comprises a heavy chain variable region and/or a variable light chain region of an antibody listed or described above.

[0162] In some embodiments, the tumor antigen is chosen from one or more of: CD19; CD 123; CD22; CD30; CD70; CD171 ; CS-1 (also referred to as CD2 subset 1, CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule- 1 (CLL-1 or CLEC12A); CD33; epidermal growth factor receptor variant III (EGFRvIII); ganglioside G2 (GD2); ganglioside GD3

5 (aNeu5Ac(2-8)aNeu5Ac(2-3)bDGalp(1-4)bDGlc(1-1)Cer); TNF receptor family member B cell maturation (BCMA); Tn antigen ((Tn Ag) or (GalNAc-Ser/Thr)); prostate-specific membrane antigen (PSMA); Receptor tyrosine kinase-like orphan receptor 1 (ROR1); Fms-Like Tyrosine Kinase 3 (FLT3); Tumor- associated glycoprotein 72 (TAG72); CD38; CD44v6; Carcinoembryonic antigen (CEA); Epithelial cell adhesion molecule (EPCAM); B7H3 (CD276);

10 KIT (CD117); Interleukin-13 receptor subunit alpha-2 (IL- 13Ra2 or CD213A2); Mesothelin; Interleukin 11 receptor alpha (IL- 11Ra); prostate stem cell antigen (PSCA); Protease Serine 21 (Testisin or PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta (PDGFR- beta); Stage- specific embryonic antigen-4 (SSEA-4); CD20; Folate receptor alpha; Receptor tyrosine-protein kinase

15 ERBB2 (Her2/neu); Mucin 1, cell surface associated (MUC-1); epidermal growth factor receptor (EGFR); neural cell adhesion molecule (NCAM); Prostase; prostatic acid phosphatase (PAP); elongation factor 2 mutated (ELF2M); Ephrin B2; fibroblast activation protein alpha (FAP); insulin-like growth factor 1 receptor (IGF-I receptor), carbonic anhydrase IX (CAIX); Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2); glycoprotein 100 (gp100);

20 oncogene fusion protein consisting of breakpoint cluster region (BCR) and Abelson murine leukemia viral oncogene homolog 1 (Abl) (bcr-abl); tyrosinase; ephrin type-A receptor 2 (EphA2); Fucosyl GM1; sialyl Lewis adhesion molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGalp(1-4)bDGlc(1-1)Cer); transglutaminase 5 (TGS5); high molecular weight-melanoma-associated antigen (HMWMAA); o-acetyl-GD2 ganglioside (OAcGD2); Folate receptor beta;

25 tumor endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R); claudin 6 (CLDN6); thyroid stimulating hormone receptor (TSHR); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); CD97; CD179a; anaplastic lymphoma kinase (ALK); Polysialic acid; placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycosphingolipid (GloboH); mammary gland

30 differentiation antigen (NY-BR-1); uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex, locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); Cancer/testis antigen 1 (NY-ESO-1); Cancer/testis antigen 2 (LAGE-la); Melanoma-

Attorney Docket No.: VSM-064WO

associated antigen 1 (MAGE-A1); ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML); sperm protein 17 (SPA17); X Antigen Family, Member 1A (XAGE1); angiopoietin-binding cell surface receptor 2 (Tie 2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1; tumor protein p53 (p53); p53 mutant; prostein; surviving; telomerase; prostate carcinoma tumor antigen- 1 (PCTA-1 or Galectin 8), melanoma antigen recognized by T cells 1 (MelanA or MART1); Rat sarcoma (Ras) mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N-Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B 1; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Tyrosinase-related protein 2 (TRP-2); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger Protein)- Like (BORIS or Brother of the Regulator of Imprinted Sites), Squamous Cell Carcinoma antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES 1); lymphocyte- specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Receptor for Advanced Glycation Endproducts (RAGE-1); renal ubiquitous 1 (RU1); renal ubiquitous 2 (RU2); legumain; human papilloma virus E6 (HPV E6); human papilloma virus E7 (HPV E7); intestinal carboxyl esterase; heat shock protein 70-2 mutated (mut hsp70-2); CD79a; CD79b; CD72; Leukocyte-associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR or CD89); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor-like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); and immunoglobulin lambda-like polypeptide 1 (IGLL1).

[0163] In some embodiments, the antigen binding domain comprises one, two, or three (*e.g.*, all three) heavy chain CDRs, HCDR1, HCDR2 and HCDR3, from an antibody listed above, and/or one, two, three (*e.g.*, all three) light chain CDRs, LCDR1, LCDR2 and LCDR3, from an antibody listed above. In embodiments, the antigen binding domain comprises a heavy chain variable region and/or a variable light chain region of an antibody listed or described above. In other embodiments, the VH and/or VL amino acid sequences may be 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% identical to the sequences described above.

Attorney Docket No.: VSM-064WO

[0164] In one aspect, the anti-tumor antigen binding domain is a fragment, *e.g.*, a single chain variable fragment (scFv). In one aspect, the anti-cancer associate antigen as described herein binding domain is an Fv, a Fab, a (Fab')₂, or a bi-functional (*e.g.* bi-specific) hybrid antibody (*e.g.*, Lanzavecchia *et al.*, Eur. J. Immunol. 17, 105 (1987)). In one aspect, the antibodies and
5 fragments thereof of the invention bind a cancer associate antigen as described herein with wild-type or enhanced affinity.

[0165] In some instances, scFvs can be prepared according to a method known in the art (see, for example, Bird *et al.*, (1988) Science 242:423-426 and Huston *et al.*, (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). ScFv molecules can be produced by linking VH and VL regions
10 together using flexible polypeptide linkers. The scFv molecules comprise a linker (*e.g.*, a Ser-Gly linker) with an optimized length and/or amino acid composition. The linker length can greatly affect how the variable regions of an scFv fold and interact. In fact, if a short polypeptide linker is employed (*e.g.*, between 5-10 amino acids) intrachain folding is prevented. Interchain folding is also required to bring the two variable regions together to form a functional epitope
15 binding site. For examples of linker orientation and size see, *e.g.*, Hollinger *et al.* 1993 Proc Natl Acad. Sci. U.S.A. 90:6444-6448, U.S. Patent Application Publication Nos. 2005/0100543, 2005/0175606, 2007/0014794, and PCT publication Nos. WO2006/020258 and WO2007/024715, which are incorporated herein by reference.

[0166] An scFv can comprise a linker of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,
20 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or more amino acid residues between its VL and VH regions. The linker sequence may comprise any naturally occurring amino acid. In some embodiments, the linker sequence comprises amino acids glycine and serine. In another embodiment, the linker sequence comprises sets of glycine and serine repeats such as (Gly₄Ser)_n, where n is a positive integer equal to or greater than 1. Variation in the linker length
25 may retain or enhance activity, giving rise to superior efficacy in activity studies.

[0167] In another aspect, the antigen binding domain is a T cell receptor ("TCR"), or a fragment thereof, for example, a single chain TCR (scTCR). Methods to make such TCRs are known in the art. See, *e.g.*, Willemsen RA *et al.*, Gene Therapy 7: 1369-1377 (2000); Zhang T *et al.*, Cancer Gene Ther 11: 487-496 (2004); Aggen *et al.*, Gene Ther. 19(4):365-74 (2012)
30 (references are incorporated herein by its entirety). For example, scTCR can be engineered that contains the Va and v β genes from a T cell clone linked by a linker (*e.g.*, a flexible peptide). This approach is very useful to cancer associated target that itself is intracellular, however, a fragment of such antigen (peptide) is presented on the surface of the cancer cells by MHC.

Transmembrane domain

[0168] With respect to the transmembrane domain, in various embodiments, a CAR can be designed to comprise a transmembrane domain that is attached to the extracellular domain of the CAR. A transmembrane domain can include one or more additional amino acids adjacent to the transmembrane region, *e.g.*, one or more amino acid associated with the extracellular region of the protein from which the transmembrane was derived (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the extracellular region) and/or one or more additional amino acids associated with the intracellular region of the protein from which the transmembrane protein is derived (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the intracellular region). In one aspect, the transmembrane domain is one that is associated with one of the other domains of the CAR. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins, *e.g.*, to minimize interactions with other members of the receptor complex. In one aspect, the transmembrane domain is capable of homodimerization with another CAR on the cell surface of a CAR-expressing cell. In a different aspect, the amino acid sequence of the transmembrane domain may be modified or substituted so as to minimize interactions with the binding domains of the native binding partner present in the same CAR.

[0169] The transmembrane domain may be derived either from a natural or from a recombinant source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. In one aspect the transmembrane domain is capable of signalling to the intracellular domain(s) whenever the CAR has bound to a target. A transmembrane domain of particular use in this invention may include at least the transmembrane region(s) of *e.g.*, the alpha, beta or zeta chain of the T-cell receptor, CD28, CD27, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154. In some embodiments, a transmembrane domain may include at least the transmembrane region(s) of, *e.g.*, KIR2DS2, OX40, CD2, CD27, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRP1), NKp44, NKp30, NKp46, CD160, IL2R beta, IL2R gamma, IL7Ra, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6

Attorney Docket No.: VSM-064WO

(NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKG2D, NKG2C, or CD19.

[0170] In some instances, the transmembrane domain can be attached to the extracellular region of the CAR, *e.g.*, the antigen binding domain of the CAR, via a hinge, *e.g.*, a hinge from a human protein. For example, in one embodiment, the hinge can be a human Ig (immunoglobulin) hinge, *e.g.*, an IgG4 hinge, or a CD8a hinge.

[0171] In one aspect, the hinge or spacer comprises an IgG4 hinge. In one aspect, the hinge or spacer comprises an IgD hinge.

[0172] In one aspect, the transmembrane domain may be recombinant, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. In one aspect a triplet of phenylalanine, tryptophan and valine can be found at each end of a recombinant transmembrane domain.

[0173] Optionally, a short oligo- or polypeptide linker, between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic region of the CAR. A glycine-serine doublet provides a particularly suitable linker.

Cytoplasmic domain

[0174] The cytoplasmic domain or region of the CAR includes an intracellular signaling domain. An intracellular signaling domain is generally responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been introduced. Examples of intracellular signaling domains for use in a CAR described herein include the cytoplasmic sequences of the T cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or variant of these sequences and any recombinant sequence that has the same functional capability. It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary and/or costimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR (primary intracellular signaling domains) and those that act in an antigen-independent manner to provide a secondary or costimulatory signal (secondary cytoplasmic domain, *e.g.*, a costimulatory domain).

[0175] A primary signaling domain regulates primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary intracellular signaling domains that act in

a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

[0176] Examples of ITAM containing primary intracellular signaling domains that are of particular use in the invention include those of TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, CD278 (also known as "ICOS"), FcεRI, DAP10, DAP12, and CD66d. In one embodiment, a CAR of the invention comprises an intracellular signaling domain, *e.g.*, a primary signaling domain of CD3-zeta, *e.g.*, a CD3-zeta sequence described herein.

[0177] In one embodiment, a primary signaling domain comprises a modified ITAM domain, *e.g.*, a mutated ITAM domain which has altered (*e.g.*, increased or decreased) activity as compared to the native ITAM domain. In one embodiment, a primary signaling domain comprises a modified ITAM-containing primary intracellular signaling domain, *e.g.*, an optimized and/or truncated ITAM-containing primary intracellular signaling domain. In an embodiment, a primary signaling domain comprises one, two, three, four or more ITAM motifs.

Costimulatory Signaling Domain

[0178] The intracellular signalling domain of the CAR can comprise the CD3-zeta signaling domain by itself or it can be combined with any other desired intracellular signaling domain(s) useful in the context of a CAR of the invention. For example, the intracellular signaling domain of the CAR can comprise a CD3 zeta chain portion and a costimulatory signaling domain. The costimulatory signaling domain refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. In one embodiment, the intracellular domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD28. In one aspect, the intracellular domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of ICOS.

[0179] A costimulatory molecule can be a cell surface molecule other than an antigen receptor or its ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. For example, CD27 costimulation has been demonstrated to enhance expansion, effector function, and survival of human CAR-T cells *in vitro* and augments human T cell persistence and antitumor activity *in vivo* (Song *et al.* Blood. 2012; 119(3):696-706). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFRR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp30, NKp44,

NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4),
5 CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, NKG2D, NKG2C and PAG/Cbp. The intracellular signaling sequences within the cytoplasmic portion of the CAR may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker, for
10 example, between 2 and 10 amino acids (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids) in length may form the linkage between intracellular signaling sequences. In one embodiment, a glycine-serine doublet can be used as a suitable linker. In one embodiment, a single amino acid, *e.g.*, an alanine, a glycine, can be used as a suitable linker.

[0180] In one aspect, the intracellular signaling domain is designed to comprise two or more,
15 *e.g.*, 2, 3, 4, 5, or more, costimulatory signaling domains. In an embodiment, the two or more, *e.g.*, 2, 3, 4, 5, or more, costimulatory signaling domains, are separated by a linker molecule, *e.g.*, a linker molecule described herein. In one embodiment, the intracellular signaling domain comprises two costimulatory signaling domains. In some embodiments, the linker molecule is a glycine residue. In some embodiments, the linker is an alanine residue.

20 [0181] In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD28. In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of 4-1BB. In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD27. Sequences of suitable domains for CAR
25 construction are known in the art. Example, non-limiting, sequences for CAR construction are presented in Table 2.

Table 2. Exemplary sequences of CAR construction

Description	Sequence
Leader sequence	MALPVTALLLPLALLHAARP (SEQ ID NO:16)

CD27 signaling domain	QRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYRKPEPA CSP (SEQ ID NO:17)
CD8 hinge domain	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:18)
CD8 transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC (SEQ ID NO:19)
4-1BB intracellular domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:20)
CD3 ζ domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:21)
CD3 ζ domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO:22)
IgG4 hinge	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLT VLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTL PPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTP PVLDSGDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQ KSLSLSLGKM (SEQ ID NO:23)
IgD hinge	RWPESPKAQASSVPTAQPPQAEGSLAKATTAPATTRNTGRGGEE KKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRD KATFTCFWGSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQ SQHSRLTLPRSLWNAGTSVTCTLNHPSLPPQRLMALREPAAQAP VKLSLNLLASSDPPEAASWLLCEVSGFSPPNILLMWLEDQREVN TSGFAPARPPPQPGSTTFWAWSVLRVPAPPSPQPATYTCWSHED SRTLLNASRSLEVS YVTDH

(SEQ ID NO:24)

Co-expression of a Second CAR

[0182] In one aspect, the CAR-expressing cell described herein can further comprise a second CAR, *e.g.*, a second CAR that includes a different antigen binding domain, *e.g.*, to the same target (*e.g.*, CD19) or a different target (*e.g.*, a target other than CD19, *e.g.*, a target described herein). In one embodiment, the CAR-expressing cell comprises a first CAR that targets a first antigen and includes an intracellular signaling domain having a costimulatory signaling domain but not a primary signaling domain, and a second CAR that targets a second, different, antigen and includes an intracellular signaling domain having a primary signaling domain but not a costimulatory signaling domain. Placement of a costimulatory signaling domain, *e.g.*, 4-1BB, CD28, CD27, OX-40 or ICOS, onto the first CAR, and the primary signaling domain, *e.g.*, CD3 zeta, on the second CAR can limit the CAR activity to cells where both targets are expressed. In one embodiment, the CAR expressing cell comprises a first CAR that includes an antigen binding domain, a transmembrane domain and a costimulatory domain and a second CAR that targets another antigen and includes an antigen binding domain, a transmembrane domain and a primary signaling domain. In another embodiment, the CAR expressing cell comprises a first CAR that includes an antigen binding domain, a transmembrane domain and a primary signaling domain and a second CAR that targets another antigen and includes an antigen binding domain to the antigen, a transmembrane domain and a costimulatory signaling domain. In one embodiment, the CAR-expressing cell comprises an XCAR described herein and an inhibitory CAR. In one embodiment, the inhibitory CAR comprises an antigen binding domain that binds an antigen found on normal cells but not cancer cells, *e.g.*, normal cells that also express X. In one embodiment, the inhibitory CAR comprises the antigen binding domain, a transmembrane domain and an intracellular domain of an inhibitory molecule. For example, the intracellular domain of the inhibitory CAR can be an intracellular domain of PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (CEACAM-1, CEACAM-3, and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (*e.g.*, TGFRbeta). In one embodiment, when the CAR-expressing cell comprises two or more different CARs, the antigen binding domains of the different CARs can be such that the antigen binding domains do not interact with one another. For example, a cell expressing a first and second CAR can have an antigen binding domain of the first CAR, *e.g.*, as a fragment, *e.g.*, an

Attorney Docket No.: VSM-064WO

scFv, that does not form an association with the antigen binding domain of the second CAR, *e.g.*, the antigen binding domain of the second CAR is a V_HH.

[0183] In some embodiments, the antigen binding domain comprises a single domain antigen binding (SDAB) molecules include molecules whose complementary determining regions are
5 part of a single domain polypeptide. Examples include, but are not limited to, heavy chain variable domains, binding molecules naturally devoid of light chains, single domains derived from conventional 4-chain antibodies, engineered domains and single domain scaffolds other than those derived from antibodies. SDAB molecules may be any of the art, or any future single domain molecules. SDAB molecules may be derived from any species including, but not limited
10 to mouse, human, camel, llama, lamprey, fish, shark, goat, rabbit, and bovine. This term also includes naturally occurring single domain antibody molecules from species other than Camelidae and sharks. In one aspect, an SDAB molecule can be derived from a variable region of the immunoglobulin found in fish, such as, for example, that which is derived from the immunoglobulin isotype known as Novel Antigen Receptor (NAR) found in the serum of shark.
15 Methods of producing single domain molecules derived from a variable region of NAR ("IgNARs") are described in WO 03/014161 and Streltsov (2005) Protein Sci. 14:2901-2909.

[0184] According to another aspect, an SDAB molecule is a naturally occurring single domain antigen binding molecule known as heavy chain devoid of light chains. Such single domain molecules are disclosed in WO 9404678 and Hamers-Casterman, C. *et al.* (1993) Nature
20 363:446-448, for example. For clarity reasons, this variable domain derived from a heavy chain molecule naturally devoid of light chain is known herein as a V_HH or nanobody to distinguish it from the conventional V_H of four chain immunoglobulins. Such a V_HH molecule can be derived from Camelidae species, for example in camel, llama, dromedary, alpaca and guanaco. Other species besides Camelidae may produce heavy chain molecules naturally devoid of light chain;
25 such V_HHs are within the scope of the invention. The SDAB molecules can be recombinant, CDR-grafted, humanized, camelized, de-immunized and/or *in vitro* generated (*e.g.*, selected by phage display).

[0185] It has also been discovered, that cells having a plurality of chimeric membrane embedded receptors comprising an antigen binding domain that interactions between the antigen
30 binding domain of the receptors can be undesirable, *e.g.*, because it inhibits the ability of one or more of the antigen binding domains to bind its cognate antigen. Accordingly, disclosed herein are cells having a first and a second non-naturally occurring chimeric membrane embedded receptor comprising antigen binding domains that minimize such interactions. Also disclosed

herein are nucleic acids encoding a first and a second non-naturally occurring chimeric membrane embedded receptor comprising an antigen binding domains that minimize such interactions, as well as methods of making and using such cells and nucleic acids. In an embodiment the antigen binding domain of one of the first and the second non- naturally occurring chimeric membrane embedded receptor, comprises an scFv, and the other comprises a single VH domain, *e.g.*, a camelid, shark, or lamprey single VH domain, or a single VH domain derived from a human or mouse sequence. In some embodiments, a composition herein comprises a first and second CAR, wherein the antigen binding domain of one of the first and the second CAR does not comprise a variable light domain and a variable heavy domain. In some embodiments, the antigen binding domain of one of the first and the second CAR is an scFv, and the other is not an scFv. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises a single VH domain, *e.g.*, a camelid, shark, or lamprey single VH domain, or a single VH domain derived from a human or mouse sequence. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises a nanobody. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises a camelid V_{HH} domain.

[0186] In some embodiments, the antigen binding domain of one of the first and the second CAR comprises an scFv, and the other comprises a single VH domain, *e.g.*, a camelid, shark, or lamprey single VH domain, or a single VH domain derived from a human or mouse sequence. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises an scFv, and the other comprises a nanobody. In some embodiments, the antigen binding domain of one of the first and the second CAR comprises an scFv, and the other comprises a camelid V_{HH} domain.

[0187] In some embodiments, when present on the surface of a cell, binding of the antigen binding domain of the first CAR to its cognate antigen is not substantially reduced by the presence of the second CAR. In some embodiments, binding of the antigen binding domain of the first CAR to its cognate antigen in the presence of the second CAR is at least 85%, 90%, 95%, 96%, 97%, 98% or 99%, *e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99% of binding of antigen binding domain of the first CAR to its cognate antigen in the absence of the second CAR.

[0188] In some embodiments, when present on the surface of a cell, the antigen binding domains of the first and the second CAR, associate with one another less than if both were scFv antigen binding domains. In some embodiments, the antigen binding domains of the first and the

second CAR, associate with one another at least 85%, 90%, 95%, 96%, 97%, 98% or 99% less than, *e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99% less than if both were scFv antigen binding domains.

Co-expression of an Agent that Enhances CAR Activity

- 5 [0189] In another aspect, the CAR-expressing cell described herein can further express another agent, *e.g.*, an agent that enhances the activity or fitness of a CAR-expressing cell. For example, in one embodiment, the agent can be an agent which inhibits a molecule that modulates or regulates, *e.g.*, inhibits, T cell function. In some embodiments, the molecule that modulates or regulates T cell function is an inhibitory molecule. Inhibitory molecules, *e.g.*, PD1, can, in some
- 10 embodiments, decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, or TGFR beta.
- 15 [0190] In embodiments, an agent, *e.g.*, an inhibitory nucleic acid, *e.g.*, a dsRNA, *e.g.*, an siRNA or shRNA; or *e.g.*, an inhibitory protein or system, *e.g.*, a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), *e.g.*, as described herein, can be used to inhibit expression of a molecule that modulates or regulates, *e.g.*, inhibits, T-cell function in the CAR- expressing
- 20 cell. In an embodiment the agent is an shRNA, *e.g.*, an shRNA described herein. In an embodiment, the agent that modulates or regulates, *e.g.*, inhibits, T-cell function is inhibited within a CAR-expressing cell. For example, a dsRNA molecule that inhibits expression of a molecule that modulates or regulates, *e.g.*, inhibits, T-cell function is linked to the nucleic acid that encodes a component, *e.g.*, all of the components, of the CAR.
- 25 [0191] In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, *e.g.*, an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, *e.g.*, an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, *e.g.*, of an inhibitory molecule such as PD1, PD-L1, CTLA4, TIM3, LAG3, VISTA, BTLA, TIGIT, LAIR1, CD 160, 2B4, CD80, CD86, B7-
- 30 H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, or TGFR beta, or a fragment of any of these (*e.g.*, at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (*e.g.*, comprising a costimulatory domain (*e.g.*, 4-

Attorney Docket No.: VSM-064WO

1BB, CD27 or CD28, *e.g.*, as described herein) and/or a primary signaling domain (*e.g.*, a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PD1 or a fragment thereof (*e.g.*, at least a portion of an extracellular domain of PD1), and a second polypeptide of an intracellular signaling domain described herein (*e.g.*, a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein). PD1 is an inhibitory member of the CD28 family of receptors that also includes CD28, CTLA-4, ICOS, and BTLA. PD-1 is expressed on activated B cells, T cells and myeloid cells (Agata *et al.* 1996 *Int. Immunol* 8:765-75). Two ligands for PD1, PD-L1 and PD-L2 have been shown to downregulate T cell activation upon binding to PD1 (Freeman *et al.* 2000 *J Exp Med* 192: 1027-34; Latchman *et al.* 2001 *Nat Immunol* 2:261-8; Carter *et al.* 2002 *Eur J Immunol* 32:634-43). PD-L1 is abundant in human cancers (Dong *et al.* 2003 *J Mol Med* 81:281-7; Blank *et al.* 2005 *Cancer Immunol. Immunother* 54:307-314; Konishi *et al.* 2004 *Clin Cancer Res* 10:5094). Immune suppression can be reversed by inhibiting the local interaction of PD1 with PD-L1.

[0192] In one embodiment, the agent comprises the extracellular domain (ECD) of an inhibitory molecule, *e.g.*, Programmed Death 1 (PD1), can be fused to a transmembrane domain and intracellular signaling domains such as 4-1BB and CD3 zeta (also referred to herein as a PD1 CAR). In one embodiment, the PD1 CAR, when used in combinations with an XCAR described herein, improves the persistence of the T cell. In one embodiment, the CAR is a PD1 CAR comprising the extracellular domain of PD1.

[0193] In another example, in one embodiment, the agent which enhances the activity of a CAR-expressing cell can be a costimulatory molecule or costimulatory molecule ligand. Examples of costimulatory molecules include MHC class I molecule, BTLA and a Toll ligand receptor, as well as OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-1BB (CD137). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83. Examples of costimulatory molecule ligands include

Attorney Docket No.: VSM-064WO

CD80, CD86, CD40L, ICOSL, CD70, OX40L, 4-1BBL, GITRL, and LIGHT. In embodiments, the costimulatory molecule ligand is a ligand for a costimulatory molecule different from the costimulatory molecule domain of the CAR. In embodiments, the costimulatory molecule ligand is a ligand for a costimulatory molecule that is the same as the costimulatory molecule domain of the CAR. In an embodiment, the costimulatory molecule ligand is 4-1BBL. In an embodiment, the costimulatory ligand is CD80 or CD86. In an embodiment, the costimulatory molecule ligand is CD70. In embodiments, a CAR-expressing immune effector cell described herein can be further engineered to express one or more additional costimulatory molecules or costimulatory molecule ligands.

10 *Co-expression of CAR with a Chemokine Receptor*

[0194] In embodiments, the CAR-expressing cell described herein, *e.g.*, CD19 CAR-expressing cell, further comprises a chemokine receptor molecule. Transgenic expression of chemokine receptors CCR2b or CXCR2 in T cells enhances trafficking to CCL2- or CXCL1-secreting solid tumors including melanoma and neuroblastoma (Craddock *et al.*, J Immunother. 15 2010 Oct; 33(8):780-8 and Kershaw *et al.*, Hum Gene Ther. 2002 Nov 1; 13(16): 1971-80). Thus, without wishing to be bound by theory, it is believed that chemokine receptors expressed in CAR-expressing cells that recognize chemokines secreted by tumors, *e.g.*, solid tumors, can improve homing of the CAR-expressing cell to the tumor, facilitate the infiltration of the CAR-expressing cell to the tumor, and enhances antitumor efficacy of the CAR-expressing cell. The chemokine receptor molecule can comprise a naturally occurring or recombinant chemokine receptor or a chemokine-binding fragment thereof. A chemokine receptor molecule suitable for expression in a CAR-expressing cell (*e.g.*, CARx) described herein include a CXC chemokine receptor (*e.g.*, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, or CXCR7), a CC chemokine receptor (*e.g.*, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, 20 CCR10, or CCR11), a CX3C chemokine receptor (*e.g.*, CX3CR1), a XC chemokine receptor (*e.g.*, XCR1), or a chemokine-binding fragment thereof. In one embodiment, the chemokine receptor molecule to be expressed with a CAR described herein is selected based on the chemokine(s) secreted by the tumor. In one embodiment, the CAR-expressing cell described herein further comprises, *e.g.*, expresses, a CCR2b receptor or a CXCR2 receptor. In an embodiment, the CAR described herein and the chemokine receptor molecule are on the same 30 vector or are on two different vectors. In embodiments where the CAR described herein and the chemokine receptor molecule are on the same vector, the CAR and the chemokine receptor

molecule are each under control of two different promoters or are under the control of the same promoter.

Nucleic Acid Constructs Encoding a CAR

[0195] The present invention also provides an immune effector cell, *e.g.*, made by a method described herein, that includes a nucleic acid molecules encoding one or more CAR constructs described herein. In one aspect, the nucleic acid molecule is provided as a messenger RNA transcript. In one aspect, the nucleic acid molecule is provided as a DNA construct. The nucleic acid molecules described herein can be a DNA molecule, an RNA molecule, or a combination thereof. In one embodiment, the nucleic acid molecule is an mRNA encoding a CAR polypeptide as described herein. In other embodiments, the nucleic acid molecule is a vector that includes any of the aforesaid nucleic acid molecules.

[0196] In one aspect, the antigen binding domain of a CAR of the invention (*e.g.*, an scFv) is encoded by a nucleic acid molecule whose sequence has been codon optimized for expression in a mammalian cell. In one aspect, entire CAR construct of the invention is encoded by a nucleic acid molecule whose entire sequence has been codon optimized for expression in a mammalian cell. Codon optimization refers to the discovery that the frequency of occurrence of synonymous codons (*i.e.*, codons that code for the same amino acid) in coding DNA is biased in different species. Such codon degeneracy allows an identical polypeptide to be encoded by a variety of nucleotide sequences. A variety of codon optimization methods is known in the art, and include, *e.g.*, methods disclosed in at least US Patent Numbers 5,786,464 and 6,114,148.

[0197] Accordingly, in one aspect, an immune effector cell, *e.g.*, made by a method described herein, includes a nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises an antigen binding domain that binds to a tumor antigen described herein, a transmembrane domain (*e.g.*, a transmembrane domain described herein), and an intracellular signaling domain (*e.g.*, an intracellular signaling domain described herein) comprising a stimulatory domain, *e.g.*, a costimulatory signaling domain (*e.g.*, a costimulatory signaling domain described herein) and/or a primary signaling domain (*e.g.*, a primary signaling domain described herein, *e.g.*, a zeta chain described herein).

[0198] The present invention also provides vectors in which a nucleic acid molecule encoding a CAR, *e.g.*, a nucleic acid molecule described herein, is inserted. Vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine

leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity. A retroviral vector may also be, *e.g.*, a gammaretroviral vector. A gammaretroviral vector may include, *e.g.*, a promoter, a packaging signal (ψ), a primer binding site (PBS), one or more (*e.g.*, two) long terminal repeats (LTR), and
5 a transgene of interest, *e.g.*, a gene encoding a CAR. A gammaretroviral vector may lack viral structural genes such as gag, pol, and env. Exemplary gammaretroviral vectors include Murine Leukemia Virus (MLV), Spleen-Focus Forming Virus (SFFV), and Myeloproliferative Sarcoma Virus (MPSV), and vectors derived therefrom. Other gammaretroviral vectors are described, *e.g.*, in Tobias Maetzig *et al.*, "Gammaretroviral Vectors: Biology, Technology and Application"
10 Viruses. 2011 Jun; 3(6): 677-713.

[0199] In another embodiment, the vector comprising the nucleic acid encoding the desired CAR is an adenoviral vector (A5/35). In another embodiment, the expression of nucleic acids encoding CARs can be accomplished using of transposons such as sleeping beauty, crispr, CAS9, and zinc finger nucleases. See below June *et al.* 2009 Nature Reviews Immunology 9.10:
15 704-716, is incorporated herein by reference.

[0200] Briefly, the expression of natural or synthetic nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration eukaryotes. Typical cloning vectors contain
20 transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

[0201] The nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression
25 vectors, replication vectors, probe generation vectors, and sequencing vectors.

[0202] Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook *et al.*, 2012, Molecular Cloning: A Laboratory Manual, volumes 1 -4, Cold Spring Harbor Press, NY), and in other virology and molecular biology manuals. Viruses, which are useful as vectors
30 include, but are not limited to, retroviruses, adenoviruses, adeno- associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (*e.g.*, WO01/96584; WO01/29058; and U.S. Pat. No. 6,326,193).

[0203] A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

[0204] Additional promoter elements, *e.g.*, enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. Exemplary promoters include the CMV IE gene, EF-1a, ubiquitin C, or phosphoglycerokinase (PGK) promoters.

[0205] An example of a promoter that is capable of expressing a CAR encoding nucleic acid molecule in a mammalian T cell is the EF1a promoter. The native EF1a promoter drives expression of the alpha subunit of the elongation factor- 1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. The EF1a promoter has been extensively used in mammalian expression plasmids and has been shown to be effective in driving CAR expression from nucleic acid molecules cloned into a lentiviral vector. See, *e.g.*, Milone *et al.*, Mol. Ther. 17(8): 1453-1464 (2009).

[0206] Another example of a promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the elongation factor- 1a promoter, the hemoglobin promoter, and the

creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or
5 turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

[0207] Another example of a promoter is the phosphoglycerate kinase (PGK) promoter. In embodiments, a truncated PGK promoter (*e.g.*, a PGK promoter with one or more, *e.g.*, 1, 2, 5,
10 10, 100, 200, 300, or 400, nucleotide deletions when compared to the wild-type PGK promoter sequence) may be desired.

[0208] A vector may also include, *e.g.*, a signal sequence to facilitate secretion, a polyadenylation signal and transcription terminator (*e.g.*, from Bovine Growth Hormone (BGH) gene), an element allowing episomal replication and replication in prokaryotes (*e.g.* SV40 origin
15 and ColEI or others known in the art) and/or elements to allow selection (*e.g.*, ampicillin resistance gene and/or zeocin marker).

[0209] In order to assess the expression of a CAR polypeptide or portions thereof, the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the
20 population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic -resistance genes, such as neo and the like.

25 [0210] Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, *e.g.*, enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the
30 recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (*e.g.*, Ui-Tei *et al.*, 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially.

In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

5 **[0211]** In embodiments, the vector may comprise two or more nucleic acid sequences encoding a CAR, *e.g.*, a CAR described herein, *e.g.*, a CD19 CAR, and a second CAR, *e.g.*, an inhibitory CAR or a CAR that specifically binds to an antigen other than CD19. In such embodiments, the two or more nucleic acid sequences encoding the CAR are encoded by a single nucleic molecule in the same frame and as a single polypeptide chain. In this aspect, the
10 two or more CARs, can, *e.g.*, be separated by one or more peptide cleavage sites, (*e.g.*, an auto-cleavage site or a substrate for an intracellular protease). Examples of peptide cleavage sites include T2A, P2A, E2A, or F2A sites.

[0212] Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, *e.g.*,
15 mammalian, bacterial, yeast, or insect cell by any method, *e.g.*, one known in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

[0213] Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and
20 the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook *et al.*, 2012, MOLECULAR CLONING: A LABORATORY MANUAL, volumes 1-4, Cold Spring Harbor Press, NY). A suitable method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

[0214] Biological methods for introducing a polynucleotide of interest into a host cell include
25 the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, *e.g.*, human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

30 **[0215]** Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

An exemplary colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (e.g., an artificial membrane vesicle). Other methods of state-of-the-art targeted delivery of nucleic acids are available, such as delivery of polynucleotides with targeted nanoparticles or other suitable sub-micron sized delivery system. In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (*in vitro*, *ex vivo* or *in vivo*). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

[0216] Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about - 20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh *et al.*, 1991

Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

5 [0217] Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the recombinant nucleic acid sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such
10 as detecting the presence or absence of a particular peptide, *e.g.*, by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

Natural Killer Cell Receptor (NKR) CARs

[0218] In an embodiment, the CAR molecule described herein comprises one or more
15 components of a natural killer cell receptor (NKR), thereby forming an NKR-CAR. The NKR component can be a transmembrane domain, a hinge domain, or a cytoplasmic domain from any of the following natural killer cell receptors: killer cell immunoglobulin-like receptor (KIR), *e.g.*, KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS 1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S 1, KIR3DL2, KIR3DL3, KIR2DP1, and
20 KIR3DP1; natural cytotoxicity receptor (NCR), *e.g.*, NKp30, NKp44, NKp46; signaling lymphocyte activation molecule (SLAM) family of immune cell receptors, *e.g.*, CD48, CD229, 2B4, CD84, NTB-A, CRACC, BLAME, and CD2F-10; Fc receptor (FcR), *e.g.*, CD16, and CD64; and Ly49 receptors, *e.g.*, LY49A, LY49C. The NKR-CAR molecules described herein may interact with an adaptor molecule or intracellular signaling domain, *e.g.*, DAP12.

25 [0219] Exemplary configurations and sequences of CAR molecules comprising NKR components are described in International Publication No. WO2014/145252, the contents of which are hereby incorporated by reference.

Split CAR

[0220] In some embodiments, the CAR-expressing cell uses a split CAR. The split CAR
30 approach is described in more detail in publications WO2014/055442 and WO2014/055657. Briefly, a split CAR system comprises a cell expressing a first CAR having a first antigen binding domain and a costimulatory domain (*e.g.*, 4-1BB), and the cell also expresses a second

CAR having a second antigen binding domain and an intracellular signaling domain (*e.g.*, CD3 zeta). When the cell encounters the first antigen, the costimulatory domain is activated, and the cell proliferates. When the cell encounters the second antigen, the intracellular signaling domain is activated and cell-killing activity begins. Thus, the CAR-expressing cell is only fully activated in the presence of both antigens.

Strategies for Regulating Chimeric Antigen Receptors

[0221] In some embodiments, a regulatable CAR (RCAR) where the CAR activity can be controlled is desirable to optimize the safety and efficacy of a CAR-therapy. There are many ways CAR activities can be regulated. For example, inducible apoptosis using, *e.g.*, a caspase fused to a dimerization domain (see, *e.g.*, Di Stasa *et al.*, N Engl. J. Med. 2011 Nov. 3; 365(18): 1673-1683), can be used as a safety switch in the CAR-therapy of the instant invention. In one embodiment, the cells (*e.g.*, T cells or NK cells) expressing a CAR of the present invention further comprise an inducible apoptosis switch, wherein a human caspase (*e.g.*, caspase 9) or a modified version is fused to a modification of the human FKB protein that allows conditional dimerization. In the presence of a small molecule, such as a rapalog (*e.g.*, AP1903, AP20187), the inducible caspase (*e.g.*, caspase 9) is activated and leads to the rapid apoptosis and death of the cells (*e.g.*, T cells or NK cells) expressing a CAR of the present invention. Examples of a caspase-based inducible apoptosis switch (or one or more aspects of such a switch) have been described in, *e.g.*, US2004040047; US20110286980; US20140255360; WO1997031899; WO2014151960; WO2014164348; WO2014197638; WO2014197638; all of which are incorporated by reference herein.

[0222] In another example, CAR-expressing cells can also express an inducible Caspase-9 (iCaspase-9) molecule that, upon administration of a dimerizer drug (*e.g.*, rimiducid (also called API 903 (Bellicum Pharmaceuticals) or AP20187 (Ariad)) leads to activation of the Caspase-9 and apoptosis of the cells. The iCaspase-9 molecule contains a chemical inducer of dimerization (CID) binding domain that mediates dimerization in the presence of a CID. This results in inducible and selective depletion of CAR-expressing cells. In some cases, the iCaspase-9 molecule is encoded by a nucleic acid molecule separate from the CAR-encoding vector(s). In some cases, the iCaspase-9 molecule is encoded by the same nucleic acid molecule as the CAR-encoding vector. The iCaspase-9 can provide a safety switch to avoid any toxicity of CAR-expressing cells. See, *e.g.*, Song *et al.* Cancer Gene Ther. 2008; 15(10):667-75; Clinical Trial Id. No. NCT02107963; and Di Stasi *et al.* N. Engl. J. Med. 2011; 365: 1673-83.

[0223] Alternative strategies for regulating the CAR-therapy of the instant invention include utilizing small molecules or antibodies that deactivate or turn off CAR activity, *e.g.*, by deleting CAR-expressing cells, *e.g.*, by inducing antibody dependent cell-mediated cytotoxicity (ADCC). For example, CAR-expressing cells described herein may also express an antigen that is

5 recognized by molecules capable of inducing cell death, *e.g.*, ADCC or complement-induced cell death. For example, CAR expressing cells described herein may also express a receptor capable of being targeted by an antibody or antibody fragment. Examples of such receptors include EpCAM, VEGFR, integrins (*e.g.*, integrins $\alpha\beta3$, $\alpha4$, $\alpha13/4\beta3$, $\alpha4\beta7$, $\alpha5\beta1$, $\alpha\nu\beta3$, $\alpha\nu$), members of the TNF receptor superfamily (*e.g.*, TRAIL-R1, TRAIL-R2), PDGF Receptor, interferon

10 receptor, folate receptor, GPNMB, ICAM-1, HLA-DR, CEA, CA-125, MUC1, TAG-72, IL-6 receptor, 5T4, GD2, GD3, CD2, CD3, CD4, CD5, CD11, CD11a/LFA-1, CD15, CD18/ITGB2, CD19, CD20, CD22, CD23/IgE Receptor, CD25, CD28, CD30, CD33, CD38, CD40, CD41, CD44, CD51, CD52, CD62L, CD74, CD80, CD125, CD147/basigin, CD152/CTLA-4, CD154/CD40L, CD195/CCR5, CD319/SLAMF7, and EGFR, and truncated

15 versions thereof (*e.g.*, versions preserving one or more extracellular epitopes but lacking one or more regions within the cytoplasmic domain).

[0224] For example, a CAR-expressing cell described herein may also express a truncated epidermal growth factor receptor (EGFR) which lacks signaling capacity but retains the epitope that is recognized by molecules capable of inducing ADCC, *e.g.*, cetuximab (ERBITUX®), such

20 that administration of cetuximab induces ADCC and subsequent depletion of the CAR-expressing cells (see, *e.g.*, WO2011/056894, and Jonnalagadda *et al.*, Gene Ther. 2013; 20(8)853-860). Another strategy includes expressing a highly compact marker/suicide gene that combines target epitopes from both CD32 and CD20 antigens in the CAR-expressing cells described herein, which binds rituximab, resulting in selective depletion of the CAR-expressing

25 cells, *e.g.*, by ADCC (see, *e.g.*, Philip *et al.*, Blood. 2014; 124(8)1277-1287). Other methods for depleting CAR-expressing cells described herein include administration of CAMPATH, a monoclonal anti-CD52 antibody that selectively binds and targets mature lymphocytes, *e.g.*, CAR-expressing cells, for destruction, *e.g.*, by inducing ADCC. In other embodiments, the CAR-expressing cell can be selectively targeted using a CAR ligand, *e.g.*, an anti-idiotypic

30 antibody. In some embodiments, the anti-idiotypic antibody can cause effector cell activity, *e.g.*, ADCC or ADC activities, thereby reducing the number of CAR-expressing cells. In other embodiments, the CAR ligand, *e.g.*, the anti-idiotypic antibody, can be coupled to an agent that induces cell killing, *e.g.*, a toxin, thereby reducing the number of CAR-expressing cells.

[0225] Alternatively, the CAR molecules themselves can be configured such that the activity can be regulated, *e.g.*, turned on and off, as described below.

[0226] In other embodiments, a CAR-expressing cell described herein may also express a target protein recognized by the T cell depleting agent. In one embodiment, the target protein is CD20 and the T cell depleting agent is an anti-CD20 antibody, *e.g.*, rituximab. In such embodiment, the T cell depleting agent is administered once it is desirable to reduce or eliminate the CAR-expressing cell, *e.g.*, to mitigate the CAR induced toxicity. In other embodiments, the T cell depleting agent is an anti-CD52 antibody, *e.g.*, alemtuzumab, as described in the Examples herein.

10 [0227] In other embodiments, an RCAR comprises a set of polypeptides, typically two in the simplest embodiments, in which the components of a standard CAR described herein, *e.g.*, an antigen binding domain and an intracellular signalling domain, are partitioned on separate polypeptides or members. In some embodiments, the set of polypeptides include a dimerization switch that, upon the presence of a dimerization molecule, can couple the polypeptides to one another, *e.g.*, can couple an antigen binding domain to an intracellular signalling domain. In one
15 embodiment, a CAR of the present invention utilizes a dimerization switch as those described in, *e.g.*, WO2014127261, which is incorporated by reference herein. Additional description and exemplary configurations of such regulatable CARs are provided herein and in, *e.g.*, paragraphs 527-551 of International Publication No. WO 2015/090229 filed March 13, 2015, which is
20 incorporated by reference in its entirety.

RNA Transfection

[0228] An immune effector cell can include a CAR encoded by a messenger RNA (mRNA). In one aspect, the mRNA encoding a CAR described herein is introduced into an immune effector cell, *e.g.*, made by a method described herein, for production of a CAR-expressing cell.

25 [0229] In one embodiment, the *in vitro* transcribed RNA CAR can be introduced to a cell as a form of transient transfection. The RNA is produced by *in vitro* transcription using a polymerase chain reaction (PCR)-generated template. DNA of interest from any source can be directly converted by PCR into a template for *in vitro* mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA,
30 phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. The desired template for *in vitro* transcription is a CAR described herein. For example, the template for the RNA CAR comprises an extracellular region comprising a single chain variable domain of an antibody to a tumor associated antigen described herein; a hinge region (*e.g.*, a hinge region

described herein), a transmembrane domain (*e.g.*, a transmembrane domain described herein such as a transmembrane domain of CD8a); and a cytoplasmic region that includes an intracellular signaling domain, *e.g.*, an intracellular signaling domain described herein, *e.g.*, comprising the signaling domain of CD3-zeta and the signaling domain of 4-1BB.

- 5 [0230] In one embodiment, the DNA to be used for PCR contains an open reading frame. The DNA can be from a naturally occurring DNA sequence from the genome of an organism. In one embodiment, the nucleic acid can include some or all of the 5' and/or 3' untranslated regions (UTRs). The nucleic acid can include exons and introns. In one embodiment, the DNA to be used for PCR is a human nucleic acid sequence. In another embodiment, the DNA to be used for
- 10 PCR is a human nucleic acid sequence including the 5' and 3' UTRs. The DNA can alternatively be an artificial DNA sequence that is not normally expressed in a naturally occurring organism. An exemplary artificial DNA sequence is one that contains portions of genes that are ligated together to form an open reading frame that encodes a fusion protein. The portions of DNA that are ligated together can be from a single organism or from more than one organism.
- 15 [0231] PCR is used to generate a template for *in vitro* transcription of mRNA which is used for transfection. Methods for performing PCR are well known in the art. Primers for use in PCR are designed to have regions that are substantially complementary to regions of the DNA to be used as a template for the PCR. "Substantially complementary," as used herein, refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are
- 20 complementary, or one or more bases are non-complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of a nucleic acid that is normally transcribed in cells (the open reading
- 25 frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a nucleic acid that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR can be generated by synthetic methods that are well known in the art. "Forward primers" are primers that contain a region of nucleotides that are substantially
- 30 complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. "Upstream" is used herein to refer to a location 5' to the DNA sequence to be amplified relative to the coding strand. "Reverse primers" are primers that contain a region of nucleotides that are substantially complementary to a double-stranded DNA template that are

downstream of the DNA sequence that is to be amplified. "Downstream" is used herein to refer to a location 3' to the DNA sequence to be amplified relative to the coding strand.

[0232] Any DNA polymerase useful for PCR can be used in the methods disclosed herein. The reagents and polymerase are commercially available from a number of sources. Chemical structures with the ability to promote stability and/or translation efficiency may also be used. The RNA in embodiments has 5' and 3' UTRs. In one embodiment, the 5' UTR is between one and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to, designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

[0233] The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the nucleic acid of interest. Alternatively, UTR sequences that are not endogenous to the nucleic acid of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the nucleic acid of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of mRNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art. In one embodiment, the 5' UTR can contain the Kozak sequence of the endogenous nucleic acid. Alternatively, when a 5' UTR that is not endogenous to the nucleic acid of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many mRNAs is known in the art. In other embodiments the 5' UTR can be 5'UTR of an RNA virus whose RNA genome is stable in cells. In other embodiments various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the mRNA.

[0234] To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In one

Attorney Docket No.: VSM-064WO

embodiment, the promoter is a T7 polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters.

[0235] RNA can be introduced into target cells using any of a number of different methods, for instance, commercially available methods which include, but are not limited to, electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, Mass.) or the Gene Pulser II (BioRad, Denver, Colo.), Multiporator (Eppendorf, Hamburg Germany), cationic liposome mediated transfection using lipofection, polymer encapsulation, peptide mediated transfection, or biolistic particle delivery systems such as "gene guns" (see, for example, Nishikawa, *et al.* Hum Gene Ther., 12(8):861-70 (2001).

Non-viral delivery methods

[0236] In some aspects, non-viral methods can be used to deliver a nucleic acid encoding a CAR described herein into a cell or tissue or a subject.

[0237] In some embodiments, the non-viral method includes the use of a transposon (also called a transposable element). In some embodiments, a transposon is a piece of DNA that can insert itself at a location in a genome, for example, a piece of DNA that is capable of self-replicating and inserting its copy into a genome, or a piece of DNA that can be spliced out of a longer nucleic acid and inserted into another place in a genome. For example, a transposon comprises a DNA sequence made up of inverted repeats flanking genes for transposition. Exemplary methods of nucleic acid delivery using a transposon include a Sleeping Beauty transposon system (SBTS) and a piggyBac (PB) transposon system. See, *e.g.*, Aronovich *et al.* Hum. Mol. Genet. 20.R1(2011):R14-20; Singh *et al.* Cancer Res. 15(2008):2961-2971; Huang *et al.* Mol. Ther. 16(2008):580-589; Grabundzija *et al.* Mol. Ther. 18(2010): 1200-1209; Kebriaei *et al.* Blood. 122.21(2013): 166; Williams. Molecular Therapy 16.9(2008): 1515-16; Bell *et al.* Nat. Protoc. 2.12(2007):3153-65; and Ding *et al.* Cell. 122.3(2005):473-83, all of which are incorporated herein by reference.

[0238] The SBTS includes two components: 1) a transposon containing a transgene and 2) a source of transposase enzyme. The transposase can transpose the transposon from a carrier plasmid (or other donor DNA) to a target DNA, such as a host cell chromosome/genome. For example, the transposase binds to the carrier plasmid/donor DNA, cuts the transposon (including

transgene(s)) out of the plasmid, and inserts it into the genome of the host cell. See, *e.g.*, Aronovich *et al.*, above.

[0239] Exemplary transposons include a pT2-based transposon. See, *e.g.*, Grabundzija *et al.* Nucleic Acids Res. 41.3(2013): 1829-47; and Singh *et al.* Cancer Res. 68.8(2008): 2961-2971, all of which are incorporated herein by reference. Exemplary transposases include a Tel/mariner- type transposase, *e.g.*, the SB 10 transposase or the SB 11 transposase (a hyperactive transposase which can be expressed, *e.g.*, from a cytomegalovirus promoter). See, *e.g.*, Aronovich *et al.*; Kebriaei *et al.*; and Grabundzija *et al.*, all of which are incorporated herein by reference. Use of the SBTS permits efficient integration and expression of a transgene, *e.g.*, a nucleic acid encoding a CAR described herein. Provided herein are methods of generating a cell, *e.g.*, T cell or NK cell, that stably expresses a CAR described herein, *e.g.*, using a transposon system such as SBTS.

[0240] In accordance with methods described herein, in some embodiments, one or more nucleic acids, *e.g.*, plasmids, containing the SBTS components are delivered to a cell (*e.g.*, T or NK cell). For example, the nucleic acid(s) are delivered by standard methods of nucleic acid (*e.g.*, plasmid DNA) delivery, *e.g.*, methods described herein, *e.g.*, electroporation, transfection, or lipofection. In some embodiments, the nucleic acid contains a transposon comprising a transgene, *e.g.*, a nucleic acid encoding a CAR described herein. In some embodiments, the nucleic acid contains a transposon comprising a transgene (*e.g.*, a nucleic acid encoding a CAR described herein) as well as a nucleic acid sequence encoding a transposase enzyme. In other embodiments, a system with two nucleic acids is provided, *e.g.*, a dual-plasmid system, *e.g.*, where a first plasmid contains a transposon comprising a transgene, and a second plasmid contains a nucleic acid sequence encoding a transposase enzyme. For example, the first and the second nucleic acids are co-delivered into a host cell.

[0241] In some embodiments, cells, *e.g.*, T or NK cells, are generated that express a CAR described herein by using a combination of gene insertion using the SBTS and genetic editing using a nuclease (*e.g.*, Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), the CRISPR/Cas system, or engineered meganuclease re-engineered homing endonucleases). In some embodiments, use of a non-viral method of delivery permits reprogramming of cells, *e.g.*, T or NK cells, and direct infusion of the cells into a subject. Advantages of non- viral vectors include but are not limited to the ease and relatively low cost of producing sufficient amounts required to meet a patient population, stability during storage, and lack of immunogenicity.

Tumor infiltrating lymphocyte therapy

[0242] In some embodiments, immune effector cells described herein comprise tumor infiltrating lymphocytes (TILs). In embodiments, TILs are isolated from a subject and expanded *ex vivo*. Methods of TIL expansion are described, for example, in US 10,398,734, incorporated
 5 by reference in its entirety. In some embodiments, TILs are enriched by selecting for a marker such as CD137. Methods of TIL isolation and enrichment are described, for example, in US10,233,425, incorporated by reference in its entirety.

[0243] The tumor microenvironment, *e.g.*, of a solid tumor, may be immunosuppressive. Tumor-infiltrating immune cells, *e.g.*, TILs, may be rendered dysfunctional by said
 10 immunosuppressive microenvironment. For example and without limitation, a tumor, *e.g.*, a solid tumor, inhibits induction of PGC1 α in tumor-infiltrating lymphocytes (TILs) through AKT signaling (Scharping, *et al.*, 2016. The Tumor Microenvironment Represses T Cell Mitochondrial Biogenesis to Drive Intratumoral T Cell Metabolic Insufficiency and Dysfunction, *Immunity* **45**, 374–388). In some embodiments, administration of a dual PI3K δ/γ , *e.g.*, duvelisib,
 15 induces mitochondrial biogenesis in tumor-infiltrating immune cells, *e.g.*, TILs. In certain embodiments, administration of a dual PI3K δ/γ , *e.g.*, duvelisib, increases transcription of PGC1 α in tumor-infiltrating immune cells, *e.g.*, TILs. In some embodiments, the tumor microenvironment is immunosuppressive and administration of a dual PI3K δ/γ , *e.g.*, duvelisib, modulates the metabolism of tumor-infiltrating immune cells, *e.g.*, TILs. Without wishing to be
 20 bound by theory, it is hypothesized that administration of of a dual PI3K δ/γ , *e.g.*, duvelisib, increases oxidative phosphorylation in the tumor-infiltrating immune cells, *e.g.*, TILs, and increases their effector function.

[0244] In some embodiments, a dual PI3K δ/γ *e.g.*, duvelisib, is administered to a subject in need thereof concurrently with TIL therapy. In some embodiments, a dual PI3K δ/γ , *e.g.*,
 25 duvelisib, is administered to a subject prior to TIL therapy. In some embodiments, a dual PI3K δ/γ , *e.g.*, duvelisib, is administered to a subject prior to collecting the subject's immune effector cells, *e.g.*, lymphocytes, *e.g.*, lymphocytes for TIL therapy.

[0245] Therapeutic TILs as described herein can be used in the treatment of cancer, immunodeficiency diseases, and infections. The cancer may be a cancer as described herein.
 30 The infection may be a bacterial, viral, or fungal infection. Treatment may further be for an inflammatory or autoimmune disease.

Methods of Manufacture/ Production

- [0246] In some embodiments, the methods disclosed herein comprise administering a dual PI3K δ / γ inhibitor to a subject, collecting the subject's immune effector cells. In some embodiments, the method further comprises transducing the cells with nucleic acid encoding a CAR, and reintroduction of the immune effector cells to the subject. In some embodiments, the PI3K δ / γ inhibitor is administered to a subject prior to collecting the immune effector cells. In some embodiments, the PI3K δ / γ inhibitor is administered to a subject, and a biomarker of effective PI3K inhibition is assessed prior to collecting the immune effector cells. In some embodiments, the PI3K δ / γ inhibitor is administered to a subject multiple times, *e.g.*, 2x, 3x, 4x, or 5x or more, prior to collecting the immune effector cells.
- 10 [0247] In some embodiments, the PI3K δ / γ inhibitor is administered before, during, and after the CAR-therapy, *e.g.*, CAR-T therapy. In some embodiments, the PI3K δ / γ inhibitor is administered prior to CAR-therapy, *e.g.*, CAR-T therapy. In some embodiments, the PI3K δ / γ inhibitor is administered concurrently with the CAR-therapy, *e.g.*, CAR-T therapy. In some embodiments, concurrent administration of PI3K δ / γ inhibitor and CAR-T therapy comprises
- 15 administration of a reaction mixture further comprising pharmaceutically acceptable excipients. In some embodiments, the PI3K δ / γ inhibitor is administered after the CAR-therapy, *e.g.*, CAR-T therapy.
- [0248] In some embodiments, the method of contacting the immune effector cells with PI3K δ / γ inhibitor occurs outside the subject's body, *e.g.*, *ex vivo* or *in vitro*. In some
- 20 embodiments, cells are treated with in culture PI3K δ / γ , *e.g.*, duvelisib. In some embodiments, cells are treated with PI3K δ / γ , *e.g.*, duvelisib, prior to, concurrently, or after transduction with a nucleic acid encoding a CAR.
- [0249] In some embodiments, the methods disclosed herein further include administering a T cell depleting agent after treatment with the cell (*e.g.*, an immune effector cell as described
- 25 herein), thereby reducing (*e.g.*, depleting) the CAR-expressing cells (*e.g.*, the CD19CAR-expressing cells). Such T cell depleting agents can be used to effectively deplete CAR-expressing cells (*e.g.*, CD19CAR-expressing cells) to mitigate toxicity. In some embodiments, the CAR-expressing cells were manufactured according to a method herein, *e.g.*, assayed (*e.g.*, before or after transfection or transduction) according to a method herein.
- 30 [0250] In some embodiments, the T cell depleting agent is administered one, two, three, four, or five weeks after administration of the cell, *e.g.*, the population of immune effector cells, described herein.

[0251] In one embodiment, the T cell depleting agent is an agent that depletes CAR-expressing cells, *e.g.*, by inducing antibody dependent cell-mediated cytotoxicity (ADCC) and/or complement-induced cell death. For example, CAR-expressing cells described herein may also express an antigen (*e.g.*, a target antigen) that is recognized by molecules capable of inducing cell death, *e.g.*, ADCC or complement-induced cell death. For example, CAR expressing cells described herein may also express a target protein (*e.g.*, a receptor) capable of being targeted by an antibody or antibody fragment. Examples of such target proteins include, but are not limited to, EpCAM, VEGFR, integrins (*e.g.*, integrins $\alpha v\beta 3$, $\alpha 4$, $\alpha I3/4\beta 3$, $\alpha 4\beta 7$, $\alpha 5\beta 1$, $\alpha v\beta 3$, αv), members of the TNF receptor superfamily (*e.g.*, TRAIL-R1, TRAIL-R2), PDGF Receptor, interferon receptor, folate receptor, GPNMB, ICAM-1, HLA-DR, CEA, CA-125, MUC1, TAG-72, IL-6 receptor, 5T4, GD2, GD3, CD2, CD3, CD4, CD5, CD11, CD11a/LFA-1, CD15, CD18/ITGB2, CD19, CD20, CD22, CD23/IgE Receptor, CD25, CD28, CD30, CD33, CD38, CD40, CD41, CD44, CD51, CD52, CD62L, CD74, CD80, CD125, CD147/basigin, CD152/CTLA-4, CD154/CD40L, CD195/CCR5, CD319/SLAMF7, and EGFR, and truncated versions thereof (*e.g.*, versions preserving one or more extracellular epitopes but lacking one or more regions within the cytoplasmic domain).

[0252] In some embodiments, the CAR expressing cell co-expresses the CAR and the target protein, *e.g.*, naturally expresses the target protein or is engineered to express the target protein. For example, the cell, *e.g.*, the population of immune effector cells, can include a nucleic acid (*e.g.*, vector) comprising the CAR nucleic acid (*e.g.*, a CAR nucleic acid as described herein) and a nucleic acid encoding the target protein.

[0253] In one embodiment, the T cell depleting agent is a CD52 inhibitor, *e.g.*, an anti-CD52 antibody molecule, *e.g.*, alemtuzumab.

[0254] In other embodiments, the cell, *e.g.*, the population of immune effector cells, expresses a CAR molecule as described herein (*e.g.*, CD19CAR) and the target protein recognized by the T cell depleting agent. In one embodiment, the target protein is CD20. In embodiments where the target protein is CD20, the T cell depleting agent is an anti-CD20 antibody, *e.g.*, rituximab.

[0255] In a further embodiment, the method further comprises transplanting a cell, *e.g.*, a hematopoietic stem cell, or bone marrow, into the subject.

[0256] In another aspect, the invention features a method of conditioning a subject prior to cell transplantation. The method includes administering to the mammal an effective amount of the cell comprising a CAR nucleic acid or polypeptide, *e.g.*, a CD19 CAR nucleic acid or

polypeptide. In some embodiments, the cell transplantation is a stem cell transplantation, *e.g.*, a hematopoietic stem cell transplantation, or a bone marrow transplantation. In other embodiments, conditioning a subject prior to cell transplantation includes reducing the number of target-expressing cells in a subject, *e.g.*, CD19-expressing normal cells or CD19-expressing cancer cells.

Activation and Expansion of Immune Effector Cells (e.g., T cells)

[0257] Immune effector cells such as T cells may be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005. Generally, a population of immune effector cells, *e.g.*, T regulatory cell depleted cells, may be expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a costimulatory molecule on the surface of the T cells. In particular, T cell populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (*e.g.*, bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4⁺ T cells or CD8⁺ T cells, an anti-CD3 antibody and an anti-CD28 antibody can be used. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diacclone, Besancon, France) can be used as can other methods commonly known in the art (Berg *et al.*, Transplant Proc. 30(8):3975-3977, 1998; Haanen *et al.*, J. Exp. Med. 190(9): 13191328, 1999; Garland *et al.*, J. Immunol Meth. 227(1-2):53-63, 1999).

[0258] In certain aspects, the primary stimulatory signal and the costimulatory signal for the T cell may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When coupled to a surface, the agents may be coupled to the same surface or to separate surfaces. Alternatively, one agent may be coupled to a surface and the other agent in solution. In one aspect, the agent providing the costimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In certain aspects, both agents can be in solution. In one aspect, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing

Attorney Docket No.: VSM-064WO

Fc receptors or an antibody or other binding agent which will bind to the agents. In this regard, see for example, U.S. Patent Application Publication Nos. 20040101519 and 20060034810 for artificial antigen presenting cells (aAPCs) that are contemplated for use in activating and expanding T cells in the present invention.

5 [0259] In one aspect, the two agents are immobilized on beads, either on the same bead or to separate beads. By way of example, the agent providing the primary activation signal is an anti-CD3 antibody or an antigen-binding fragment thereof and the agent providing the costimulatory signal is an anti-CD28 antibody or antigen-binding fragment thereof; and both agents are co -

10 immobilized to the same bead in equivalent molecular amounts. In one aspect, a 1:1 ratio of each antibody bound to the beads for CD4⁺ T cell expansion and T cell growth is used. In certain aspects of the present invention, a ratio of anti CD3:CD28 antibodies bound to the beads is used such that an increase in T cell expansion is observed as compared to the expansion observed using a ratio of 1:1. In one particular aspect an increase of from about 1 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In one aspect, the ratio of

15 CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In one aspect, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, i.e., the ratio of CD3:CD28 is less than one. In certain aspects, the ratio of anti-CD28 antibody to anti CD3 antibody bound to the beads is greater than 2: 1. In one particular aspect, a 1:100 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:75 CD3:CD28

20 ratio of antibody bound to beads is used. In a further aspect, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet one aspect, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

25 [0260] Ratios of particles to cells from 1:500 to 500:1 and any integer values in between may be used to stimulate T cells or other target cells. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. For example, small sized beads could only bind a few cells, while larger beads could bind many. In certain aspects the ratio of cells to particles ranges from 1:100 to 100:1 and any integer values

30 in-between and in further aspects the ratio comprises 1:9 to 9:1 and any integer values in between, can also be used to stimulate T cells. The ratio of anti-CD3- and anti-CD28- coupled particles to T cells that result in T cell stimulation can vary as noted above, however certain suitable values include 1:100, 1:50, 1:40, 1:30, 1:20, 1: 10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:

Attorney Docket No.: VSM-064WO

1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, and 15:1 with one suitable ratio being at least 1:1 particles per T cell. In one aspect, a ratio of particles to cells of 1:1 or less is used. In one particular aspect, a suitable particle: cell ratio is 1:5. In further aspects, the ratio of particles to cells can be varied depending on the day of stimulation. For example, in one aspect, the ratio of particles to cells is from 1:1 to 10:1 on the first day and additional particles are added to the cells every day or every other day thereafter for up to 10 days, at final ratios of from 1:1 to 1:10 (based on cell counts on the day of addition). In one particular aspect, the ratio of particles to cells is 1:1 on the first day of stimulation and adjusted to 1:5 on the third and fifth days of stimulation. In one aspect, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:5 on the third and fifth days of stimulation. In one aspect, the ratio of particles to cells is 2:1 on the first day of stimulation and adjusted to 1:10 on the third and fifth days of stimulation. In one aspect, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:10 on the third and fifth days of stimulation. One of skill in the art will appreciate that a variety of other ratios may be suitable for use in the present invention. In particular, ratios will vary depending on particle size and on cell size and type. In one aspect, the most typical ratios for use are about 1:1, 2:1 and 3:1 on the first day.

[0261] In one embodiment, cells transduced with a nucleic acid encoding a CAR, *e.g.*, a CAR described herein, *e.g.*, a CD19 CAR described herein, are expanded, *e.g.*, by a method described herein. In one embodiment, the cells are expanded in culture for a period of several hours (*e.g.*, about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 18, 21 hours) to about 14 days (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days). In one embodiment, the cells are expanded for a period of 4 to 9 days. In one embodiment, the cells are expanded for a period of 8 days or less, *e.g.*, 7, 6 or 5 days. In one embodiment, the cells are expanded in culture for 5 days, and the resulting cells are more potent than the same cells expanded in culture for 9 days under the same culture conditions. Potency can be defined, *e.g.*, by various T cell functions, *e.g.* proliferation, target cell killing, cytokine production, activation, migration, or combinations thereof. In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, expanded for 5 days show at least a one, two, three or four fold increase in cells doublings upon antigen stimulation as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, the cells expressing a CD19 CAR described herein, are expanded in culture for 5 days, and the resulting cells exhibit higher proinflammatory cytokine production, *e.g.*, IFN- γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, *e.g.*, a CD19 CAR cell described herein, expanded for 5 days show at least a one, two, three, four, five, tenfold or more increase in pg/ml of

proinflammatory cytokine production, *e.g.*, IFN- γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions.

[0262] Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell culture include an appropriate media (*e.g.*, Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF β , and TNF- α or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V, DMEM, MEM, a-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum- free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37 °C) and atmosphere (*e.g.*, air plus 5% CO₂).

[0263] In one embodiment, the cells are expanded in an appropriate media that includes one or more interleukin that result in at least a 200-fold (*e.g.*, 200-fold, 250-fold, 300-fold, 350-fold) increase in cells over a 14 day expansion period, *e.g.*, as measured by a method described herein such as flow cytometry. In one embodiment, the cells are expanded in the presence IL-15 and/or IL-7.

[0264] In embodiments, methods described herein, *e.g.*, CAR-expressing cell manufacturing methods, comprise removing T regulatory cells, *e.g.*, CD25+ T cells, from a cell population, *e.g.*, using an anti-CD25 antibody, or fragment thereof, or a CD25-binding ligand, IL-2.

[0265] Methods of removing T regulatory cells, *e.g.*, CD25+ T cells, from a cell population are described herein. In embodiments, the methods, *e.g.*, manufacturing methods, further comprise contacting a cell population (*e.g.*, a cell population in which T regulatory cells, such as CD25+ T cells, have been depleted; or a cell population that has previously contacted an anti-CD25 antibody, fragment thereof, or CD25-binding ligand) with IL-15 and/or IL-7. For example, the cell population (*e.g.*, that has previously contacted an anti-CD25 antibody, fragment thereof, or CD25-binding ligand) is expanded in the presence of IL-15 and/or IL-7.

[0266] In some embodiments a CAR-expressing cell described herein is contacted with a composition comprising a interleukin- 15 (IL-15) polypeptide, a interleukin- 15 receptor alpha (IL-15Ra) polypeptide, or a combination of both a IL-15 polypeptide and a IL-15Ra polypeptide *e.g.*, hetIL-15, during the manufacturing of the CAR-expressing cell, *e.g.*, *ex vivo*. In
5 embodiments, a CAR-expressing cell described herein is contacted with a composition comprising a IL-15 polypeptide during the manufacturing of the CAR-expressing cell, *e.g.*, *ex vivo*. In embodiments, a CAR-expressing cell described herein is contacted with a composition comprising a combination of both a IL-15 polypeptide and a IL-15 Ra polypeptide during the manufacturing of the CAR-expressing cell, *e.g.*, *ex vivo*. In embodiments, a CAR-expressing cell
10 described herein is contacted with a composition comprising hetIL-15 during the manufacturing of the CAR-expressing cell, *e.g.*, *ex vivo*.

[0267] T cells that have been exposed to varied stimulation times may exhibit different characteristics. For example, typical blood or apheresed peripheral blood mononuclear cell products have a helper T cell population (TH, CD4+) that is greater than the cytotoxic or
15 suppressor T cell population (TC, CD8+). *Ex vivo* expansion of T cells by stimulating CD3 and CD28 receptors produces a population of T cells that prior to about days 8-9 consists predominately of TH cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of TC cells. Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of TH cells may be
20 advantageous. Similarly, if an antigen- specific subset of TC cells has been isolated it may be beneficial to expand this subset to a greater degree.

[0268] Further, in addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated T cell product for specific
25 purposes. Once a CAR described herein is constructed, various assays can be used to evaluate the activity of the molecule, such as but not limited to, the ability to expand T cells following antigen stimulation, sustain T cell expansion in the absence of re-stimulation, and anti-cancer activities in appropriate *in vitro* and animal models.

[0269] *In vitro* expansion of CAR-T cells following antigen stimulation can be measured by
30 flow cytometry. For example, a mixture of CD4⁺ and CD8⁺ T cells are stimulated with aCD3/aCD28 aAPCs followed by transduction with lentiviral vectors expressing GFP under the control of the promoters to be analyzed. Exemplary promoters include the CMV IE gene, EF-1a, ubiquitin C, or phosphoglycerokinase (PGK) promoters. GFP fluorescence is evaluated on day 6

Attorney Docket No.: VSM-064WO

of culture in the CD4⁺ and/or CD8⁺ T cell subsets by flow cytometry. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009). Alternatively, a mixture of CD4⁺ and CD8⁺ T cells are stimulated with aCD3/aCD28 coated magnetic beads on day 0, and transduced with CAR on day 1 using a bicistronic lentiviral vector expressing CAR along with eGFP using a 2A
5 ribosomal skipping sequence. Cultures are re-stimulated with either a cancer associated antigen as described herein. GFP⁺ T cells are enumerated by flow cytometry using bead-based counting. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009).

[0270] Sustained CAR-T cell expansion in the absence of re-stimulation can also be measured. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009). Briefly, mean T
10 cell volume (fl) is measured on day 8 of culture using a Coulter Multisizer III particle counter, a Nexcelom Cellometer Vision or Millipore Scepter, following stimulation with CD3/CD28 coated magnetic beads on day 0, and transduction with the appropriate CAR on day 1.

[0271] Animal models can also be used to measure CAR-expressing cell activity, *e.g.*, as described in paragraph 698 of International Application WO2015/142675, filed March 13, 2015,
15 which is herein incorporated by reference in its entirety. Dose dependent CAR-treatment response can be evaluated, *e.g.*, as described in paragraph 699 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety.

[0272] Assessment of cell proliferation and cytokine production has been previously described, as described in paragraph 700 of International Application WO2015/142675, filed
20 March 13, 2015, which is herein incorporated by reference in its entirety.

[0273] Cytotoxicity can be assessed by a standard ⁵¹Cr-release assay, *e.g.*, as described in paragraph 701 of International Application WO2015/142675, filed March 13, 2015, which is
herein incorporated by reference in its entirety. Cytotoxicity can also be assessed by measuring changes in adherent cell's electrical impedance, *e.g.*, using an xCELLigence real time cell
25 analyzer (RTCA). In some embodiments, cytotoxicity is measured at multiple time points.

[0274] Imaging technologies can be used to evaluate specific trafficking and proliferation of CARs in tumor-bearing animal models, *e.g.*, as described in paragraph 702 of International Application WO2015/142675, which is herein incorporated by reference in its entirety.

[0275] Other assays, including those described in the Example section herein as well as those
30 that are known in the art can also be used to evaluate the CARs described herein.

[0276] Alternatively, or in combination to the methods disclosed herein, methods and compositions for one or more of: detection and/or quantification of CAR-expressing cells (*e.g.*,

in vitro or *in vivo* (*e.g.*, clinical monitoring)); immune cell expansion and/or activation; and/or CAR-specific selection, that involve the use of a CAR ligand, are disclosed. In one exemplary embodiment, the CAR ligand is an antibody that binds to the CAR molecule, *e.g.*, binds to the extracellular antigen binding domain of CAR (*e.g.*, an antibody that binds to the antigen binding domain, *e.g.*, an anti-idiotypic antibody; or an antibody that binds to a constant region of the extracellular binding domain). In other embodiments, the CAR ligand is a CAR antigen molecule (*e.g.*, a CAR antigen molecule as described herein).

[0277] In one aspect, a method for detecting and/or quantifying CAR-expressing cells is disclosed. For example, the CAR ligand can be used to detect and/or quantify CAR-expressing cells *in vitro* or *in vivo* (*e.g.*, clinical monitoring of CAR-expressing cells in a patient, or dosing a patient). The method includes: providing the CAR ligand (optionally, a labelled CAR ligand, *e.g.*, a CAR ligand that includes a tag, a bead, a radioactive or fluorescent label); acquiring the CAR-expressing cell (*e.g.*, acquiring a sample containing CAR-expressing cells, such as a manufacturing sample or a clinical sample); contacting the CAR-expressing cell with the CAR ligand under conditions where binding occurs, thereby detecting the level (*e.g.*, amount) of the CAR-expressing cells present. Binding of the CAR-expressing cell with the CAR ligand can be detected using standard techniques such as FACS, ELISA and the like.

[0278] In certain embodiments, the CAR ligand is present on a substrate (*e.g.*, is immobilized or attached to a substrate, *e.g.*, a non-naturally occurring substrate). In some embodiments, the substrate is a non-cellular substrate. The non-cellular substrate can be a solid support chosen from, *e.g.*, a plate (*e.g.*, a microtiter plate), a membrane (*e.g.*, a nitrocellulose membrane), a matrix, a chip or a bead. In embodiments, the CAR ligand is present in the substrate (*e.g.*, on the substrate surface). The CAR ligand can be immobilized, attached, or associated covalently or non-covalently (*e.g.*, cross-linked) to the substrate. In one embodiment, the CAR ligand is attached (*e.g.*, covalently attached) to a bead. In embodiments, the immune cell population can be expanded *in vitro* or *ex vivo*. The method can further comprise culturing the population of immune cells in the presence of the ligand of the CAR molecule, *e.g.*, using any of the methods described herein.

[0279] In other embodiments, the method of expanding and/or activating the cells further comprises addition of a second stimulatory molecule, *e.g.*, CD28. For example, the CAR ligand and the second stimulatory molecule can be immobilized to a substrate, *e.g.*, one or more beads, thereby providing increased cell expansion and/or activation.

Attorney Docket No.: VSM-064WO

[0280] In yet another aspect, a method for selecting or enriching for a CAR expressing cell is provided. The method includes contacting the CAR expressing cell with a CAR ligand as described herein; and selecting the cell on the basis of binding of the CAR ligand.

5 [0281] In yet other embodiments, a method for depleting, reducing and/or killing a CAR expressing cell is provided. The method includes contacting the CAR expressing cell with a CAR ligand as described herein; and targeting the cell on the basis of binding of the CAR ligand, thereby reducing the number, and/or killing, the CAR-expressing cell. In one embodiment, the CAR ligand is coupled to a toxic agent (*e.g.*, a toxin or a cell ablative drug).

10 [0282] Exemplary anti-CAR antibodies that can be used in the methods disclosed herein are described, *e.g.*, in WO 2014/190273 and by Jena *et al.*, "Chimeric Antigen Receptor (CAR)-Specific Monoclonal Antibody to Detect CD19-Specific T cells in Clinical Trials", PLOS March 2013 8:3 e57838, the contents of which are incorporated by reference herein.

[0283] In some embodiments, one or more CAR-expressing cells as disclosed herein can be administered or delivered to the subject via a biopolymer scaffold, *e.g.*, a biopolymer implant.
15 Biopolymer scaffolds can support or enhance the delivery, expansion, and/or dispersion of the CAR-expressing cells described herein. A biopolymer scaffold comprises a biocompatible (*e.g.*, does not substantially induce an inflammatory or immune response) and/or a biodegradable polymer that can be naturally occurring or synthetic.

Pharmaceutical compositions and treatments

20 [0284] In some aspects, the disclosure provides a method of treating a patient, comprising administering CAR-expressing cells produced as described herein, optionally in combination with one or more other therapies. In some aspects, the disclosure provides a method of treating a patient, comprising administering a reaction mixture comprising CAR-expressing cells as described herein, optionally in combination with one or more other therapies. In some aspects,
25 the disclosure provides a method of shipping or receiving a reaction mixture comprising CAR-expressing cells as described herein. In some aspects, the disclosure provides a method of treating a patient, comprising receiving a CAR-expressing cell that was produced as described herein, and further comprising administering the CAR-expressing cell to the patient, optionally in combination with one or more other therapies. In some aspects, the disclosure provides a
30 method of treating a patient, comprising producing a CAR-expressing cell as described herein, and further comprising administering the CAR-expressing cell to the patient, optionally in combination with one or more other therapies. The other therapy may be, *e.g.*, a cancer therapy such as chemotherapy.

[0285] In an embodiment, cells expressing a CAR described herein are administered to a subject in combination with a molecule that decreases the T_{REG} cell population. Methods that decrease the number of T_{REG} cells are known in the art and include, *e.g.*, CD25 depletion, cyclophosphamide administration, and modulating GITR function. Without wishing to be bound by theory, it is believed that reducing the number of T_{REG} cells in a subject prior to apheresis or prior to administration of a CAR-expressing cell described herein reduces the number of unwanted immune cells (*e.g.*, Tregs) in the tumor microenvironment and reduces the subject's risk of relapse.

[0286] The methods described herein can further include formulating a CAR-expressing cell in a pharmaceutical composition. Pharmaceutical compositions may comprise a CAR-expressing cell, *e.g.*, a plurality of CAR-expressing cells, as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions can be formulated, *e.g.*, for intravenous administration.

[0287] In one embodiment, the pharmaceutical composition is substantially free a contaminant, *e.g.*, selected from the group consisting of endotoxin, mycoplasma, replication competent lentivirus (RCL), p24, VSV-G nucleic acid, HIV gag, residual anti-CD3/anti-CD28 coated beads, mouse antibodies, pooled human serum, bovine serum albumin, bovine serum, culture media components, vector packaging cell or plasmid components, a bacterium and a fungus. In one embodiment, the bacterium is at least one selected from the group consisting of *Alcaligenes faecalis*, *Candida albicans*, *Escherichia coli*, *Haemophilus influenza*, *Neisseria meningitides*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumonia*, and *Streptococcus pyogenes* group A.

[0288] In some embodiments, a dose of CAR cells (*e.g.*, CD19 CAR cells) comprises about 1×10^6 , 1.1×10^6 , 2×10^6 , 3.6×10^6 , 5×10^6 , 1×10^7 , 1.8×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , or 5×10^{10} cells/kg. In some embodiments, a dose of CAR cells (*e.g.*, CD19 CAR cells) comprises at least about 1×10^6 , 1.1×10^6 , 2×10^6 , 3.6×10^6 , 5×10^6 , 1×10^7 , 1.8×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , or 5×10^8 cells/kg. In some embodiments, a dose of CAR cells (*e.g.*, CD 19 CAR cells) comprises up to about 1×10^6 , 1.1×10^6 , 2×10^6 , 3.6×10^6 , 5×10^6 , 1×10^7 , 1.8×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , or 5×10^8 cells/kg. In some embodiments, a dose of CAR cells

Attorney Docket No.: VSM-064WO

(e.g., CD19 CAR cells) comprises about 1.1×10^6 - 1.8×10^7 cells/kg. In some embodiments, a dose of CAR cells (e.g., CD19 CAR cells) comprises about 1×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , 5×10^8 , 1×10^9 , 2×10^9 , or 5×10^9 cells. In some embodiments, a dose of CAR cells (e.g., CD19 CAR cells) comprises at least about 1×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , 5×10^8 , 1×10^9 , 2×10^9 , or 5×10^9 cells. In some embodiments, a dose of CAR cells (e.g., CD19 CAR cells) comprises up to about 1×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , 5×10^8 , 1×10^9 , 2×10^9 , or 5×10^9 cells.

[0289] In certain aspects, it may be desired to administer activated immune effector cells (e.g., T cells, NK cells) to a subject and then subsequently withdraw blood or have an apheresis performed, activate immune effector cells (e.g., T cells, NK cells) therefrom, and reinfuse the patient with these activated and expanded immune effector cells (e.g., T cells, NK cells). This process can be carried out multiple times every few weeks. In certain aspects, immune effector cells (e.g., T cells, NK cells) can be activated from blood draws of from 10cc to 400cc. In certain aspects, immune effector cells (e.g., T cells, NK cells) are activated from blood draws of 20cc, 30cc, 40cc, 50cc, 60cc, 70cc, 80cc, 90cc, or 100cc.

[0290] The administration of the subject compositions may be carried out in any convenient manner. The compositions described herein may be administered to a patient trans arterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally, e.g., by intradermal or subcutaneous injection. The compositions of immune effector cells (e.g., T cells, NK cells) may be injected directly into a tumor, lymph node, or site of infection.

EXAMPLES

[0291] The invention now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and is not intended to limit the invention.

Example 1: *Ex vivo* treatment of whole blood with PI3K inhibitors

[0292] The relative effects of different PI3K inhibitors on PI3K- δ and PI3K- γ cell signaling downstream of LPS and fMLP, respectively, were assessed in whole blood using phosphorylated (p)-Akt serine-473 as the readout. Briefly, LPS-stimulated monocytes and fMLP-stimulated monocytes were cultured in the presence of 2 $\mu\text{g/mL}$ LPS or 50 ng/mL fMLP, respectively. After 10 minutes of stimulation, red blood cells were lysed and remaining leukocytes were fixed,

permeabilized and stained with CD45, CD3, CD45RA, CD14, CD19 and p-Akt S473 (D9E clone) antibodies. Data was acquired on an LSRII cytometer (BD Biosciences) equipped with at least 4 lasers (405, 488, 561, and 640 nm). Monocytes were gated and the percentage of positive p-Akt monocytes was measured. IC₅₀ dose curves were generated and IC₅₀ values calculated.

- 5 [0293] In order to investigate the dual PI3K- δ and PI3K- γ inhibition of duvelisib, the potency of duvelisib was first tested against known inhibitors of PI3K- δ (idelalisib and umbralisib) and PI3K- γ (IPI-549). Whole blood potencies against PI3K- δ and PI3K- γ were measured through inhibition of p-Akt in LPS-stimulated monocytes and fMLP-stimulated monocytes, respectively. Whole blood assay IC₅₀ values, which encompass enzyme inhibition, cell penetration, and
- 10 protein binding of inhibitors, are related to reported clinical plasma exposures of each agent at recommended phase II dose (RP2D). As shown in **FIG. 1 and FIG. 2**, only duvelisib inhibited both PI3K- δ and PI3K- γ at clinically achievable exposures (IC₅₀: PI3K- δ , 0.4 ± 0.1 μ M; PI3K- γ , 1.6 ± 0.2 μ M), and as expected, idelalisib and IPI-549 demonstrated single isoform inhibition of PI3K- δ and PI3K- γ , respectively. Umbralisib, however, was not capable of PI3K- δ inhibition at
- 15 clinically achievable exposures. Tenalisib, although reported to be a dual PI3K- δ and PI3K- γ inhibitor, did not meaningfully inhibit PI3K- δ nor PI3K- γ in these whole blood assays. These data are summarized in **Table 3**. Thus, these results confirmed that duvelisib is a dual PI3K- δ and PI3K- γ inhibitor whereas idelalisib and IPI-549 are single PI3K- δ and PI3K- γ isoform inhibitors, respectively.

20 **Table 3.** Summary of whole blood assay inhibitor data

Drug	Mechanism of Action	PI3K δ IC ₅₀ (μ M)	PI3K γ IC ₅₀ (μ M)	C _{max} (ss) (μ M)
Duvelisib	Dual PI3K δ/γ inhibitor	0.4 ± 0.1	1.6 ± 0.2	2.5
Idelalisib	PI3K δ inhibitor	1.0 ± 0.2	9.4 ± 2.3	4.8
IPI-549	PI3K γ inhibitor	12 ± 0.5	0.5 ± 0.2	9.1
Umbralisib		25 ± 8	55 ± 16	9.2
Tenalisib		>50	No inhibition	

Example 2: *PI3K inhibitor treatment optimizes human T cells/CAR-T and requires dual inhibition of PI3K- δ and PI3K- γ*

- [0294] As shown in **FIG. 3A**, duvelisib induces a 3 to 4-fold increase in T cell expansion *ex vivo* using T cells from human donors or CLL patients. Importantly, it was found that dual PI3K- δ/γ inhibition was necessary for the maximal effect. The maximal effect of duvelisib on T cell expansion was observed at a 40 nM concentration, which is sufficient for inhibition of both PI3K- δ (cellular IC₅₀ = 0.36 nM) and PI3K- γ (cellular IC₅₀ = 19.6 nM). As shown in **FIG. 3B**, the maximal effect of idelalisib was observed at 1 μ M concentration, which is high enough to inhibit both PI3K- δ (cellular IC₅₀ = 4.9 nM) and PI3K- γ (cellular IC₅₀ = 520 nM). These data indicate that dual inhibition of both PI3K- δ and PI3K- γ is necessary to optimize T cell expansion.
- 10 [0295] It was further determined that duvelisib treatment reduces expression of T cell exhaustion markers (*e.g.*, TIM-3, LAG-3) and this also requires dual inhibition of PI3K- δ and PI3K- γ for maximal effect (**FIG. 4**). Effects of duvelisib on CD4 expression are shown in **FIG. 4A**, effects on CD8 expression are shown in **FIG. 4B**, and effects on LAG3 are shown in **FIG. 4C**. Furthermore, duvelisib, at a concentration that inhibits both PI3K- δ and PI3K- γ (200 nM), increases the proportion of early memory T cell populations in T cells taken from CLL patients (**FIG. 5B**), as compared to T cell populations maintained under standard culture conditions (**FIG. 5A**). These early memory T cells populations have been associated with CAR-T cells that induce complete remissions. Furthermore, addition of duvelisib during generation of CD19 CAR-T cells increases the ratio of CD8/CD4 CAR-T cells (**FIG. 6A**) and the cytotoxic activity of CAR-T
- 15 cells to kill tumor cells (**FIG. 6B**).
- 20

Example 3: *Dual inhibition of PI3K- δ and PI3K- γ can be accomplished in patients with duvelisib but not with idelalisib treatment*

- [0296] As described in Example 1, whole blood assays were used to demonstrate duvelisib inhibition of both PI3K- δ (whole blood IC₅₀ = 0.4 μ M) and PI3K- γ (whole blood IC₅₀ = 1.6 μ M) at clinically achievable exposures. In contrast, the recommended phase 2 dose for idelalisib is sufficient to inhibit PI3K- δ (whole blood IC₅₀ = 1 μ M) but is not sufficient to inhibit PI3K- γ (whole blood IC₅₀ = 9.4 μ M). For comparison, IPI-549 is a PI3K- γ specific inhibitor, which can inhibit PI3K- γ but not PI3K- δ at clinically achievable exposures. These data demonstrate that only duvelisib, and not idelalisib, can inhibit both PI3K- δ and PI3K- γ at clinically achievable
- 25
- 30 exposures.

- [0297] In follicular lymphoma patients, the same PI3K- δ and PI3K- γ specific assays as described *supra* were used to assess the degree of target inhibition following oral treatment with

Attorney Docket No.: VSM-064WO

25 mg of duvelisib. As shown in **FIG. 7A** and **FIG. 7B**, duvelisib induced approximately 90-95% inhibition of PI3K- δ at 1 and 4 hours after dosing. At cycle 2 Day 1 pre, a timepoint corresponding to the 12 hour point when the next dose is to be given, the degree of inhibition is decreased; however, inhibition is re-established with the administration of the next dose.

5 Therefore, twice daily dosing (BID) of patients with duvelisib provides optimal inhibition.

[0298] Similarly to the inhibition observed for PI3K- γ , 25 mg of duvelisib induced approximately 60-70% inhibition of PI3K- γ at 1 and 4 hours, and again the degree of inhibition is decreased at cycle 2 Day 1 pre. Accordingly, BID should then re-establish inhibition in this 60-70% range.

10 [0299] Taken together, these clinical pharmacodynamic data suggest that duvelisib is an efficient inhibitor of both PI3K- δ and PI3K- γ in patients to achieve the desired effects on T cell and CAR-T phenotype that were observed *in vitro*.

INCORPORATION BY REFERENCE

[0300] Unless stated to the contrary, the entire disclosure of each of the patent documents and scientific articles referred to herein is incorporated by reference for all purposes.

EQUIVALENTS

15 [0301] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the
20 claims are intended to be embraced therein.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of creating a population of immune effector cells, the method comprising:
 - administering a dual PI3K δ/γ inhibitor to a subject; and
 - 5 collecting a sample of a subjects' immune effector cells.
2. The method of claim 1, further comprising introducing a nucleic acid into the immune effector cells, resulting in expression of chimeric antigen receptors (CAR).
3. The method of claim 1 or 2, further comprising administering the immune effector cells to the subject in need thereof.
- 10 4. The method of any one of claims 1-3, wherein the population of immune effector cells are T cells.
5. The method of any one of claims 1-3, wherein the population of immune effector cells are tumor infiltrating lymphocytes.
6. The method of claim 4, wherein the T cells express a chimeric antigen receptor comprising
 - 15 an antigen binding-fragment directed against a tumor antigen, a hinge or transmembrane region, and an intracellular T-cell signaling component.
7. The method of claim 6, wherein the tumor antigen is selected from the list comprising CD19, BCMA, CD123, CD20, CD22, CD70, and CD37.
8. The method of claim 7, wherein the tumor antigen is CD19.
- 20 9. The method of any one of claims 1-8, wherein the immune effector cells are autologous.
10. The method of any one of claims 1-8, wherein the immune effector cells are allogeneic.
11. The method of any one of claims 1-10, wherein the dual PI3K δ/γ inhibitor is duvelisib.
12. The method of any one of claims 4-11, wherein the population of T cells comprises early memory T cells or non-exhausted early memory T cells.
- 25 13. The method of claim 12, wherein the early memory T cells are CD27⁺ and/or CD45RO^{dim/ne}.

Attorney Docket No.: VSM-064WO

14. The method of claim 12, wherein the non-exhausted early memory T cells are PD-1 negative, CD27^{hi}, CCR7^{hi}, CD45RO^{dim/neg}, TIM3^{dim/neg}, and/or LAG3^{dim/neg}.
15. The method of any one of claims 12-14, wherein the population of T cells has an increased amount of about 5- about 10%, about 10- about 20%, about 20- about 30%, about 30- about 50%, about 50- about 70%, or about 70- about 90% more early memory T cells or non-exhausted early memory T cells compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ / γ inhibitor.
16. The method of any one of claims 4-15, wherein the T cells display an increased amount of about 5- about 10%, about 10- about 20%, about 20- about 30%, about 30- about 50%, about 50- about 70%, or about 70- about 90% more CD8/CD4 positive T cells compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ / γ inhibitor.
17. The method of claim 16, wherein the increased amount comprises about 40% more CD8/CD4 positive T cells compared to an otherwise similar population of T cells in a subject not administered a dual PI3K δ / γ inhibitor.
18. The method of any one of claims 1-17, wherein the administering the dual PI3K δ / γ inhibitor increases mitochondrial area within the immune effector cells.
19. The method of claim 18, wherein the increase in mitochondrial area relative to total cell area is by 1-fold, 2-fold, 3-fold, 4-fold, or 5-fold compared to an otherwise similar population of immune effector cells in a subject not administered a dual PI3K δ / γ inhibitor.
20. The method of any one of claims 1-19, wherein the dual PI3K δ / γ inhibitor is duvelisib.
21. The method of any one of claims 1-20, wherein the dual PI3K δ / γ inhibitor is administered twice daily (BID) to the subject.
22. A method of treating a subject having cancer, the method comprising (i) administering to the subject a dual PI3K δ / γ inhibitor, and (ii) administering to the subject a therapeutically effective amount of CAR-expressing immune effector cells.
23. The method of claim 22, wherein the dual PI3K δ / γ inhibitor is administered prior to administration of CAR-expressing immune effector cells.

Attorney Docket No.: VSM-064WO

24. The method of claim 22, wherein the dual PI3K δ / γ inhibitor is administered concurrently with administration of CAR-expressing immune effector cells.
25. The method of claim 22, wherein the dual PI3K δ / γ inhibitor is administered subsequent to administration of CAR-expressing immune effector cells.
- 5 26. The method of any one of claims 22-25, wherein the dual PI3K δ / γ inhibitor is duvelisib.
27. The method of any one of claims 22-26, wherein the CAR-expressing immune effector cells are CAR-T cells.
28. The method of claim 27, wherein the CAR-T cells are CD19 CAR-T cells.
29. The method of claim 28, wherein the CD19 CAR-T cells are administered as tisagenlecleucel,
10 axicabtagene ciloleucel, or lisocabtagene maraleucel.
30. The method of any one of claims 26-29, wherein the duvelisib is administered at a dose sufficient to increase populations of early memory CAR-T cells in the subject.
31. The method of any one of claims 26-30, wherein the duvelisib is administered twice daily (BID) to the subject.
- 15 32. The method of any one of claims 22-31, wherein the method is used in combination with a second cancer treatment.
33. The method of claim 32, wherein the second cancer treatment is a chemotherapy.

1/5

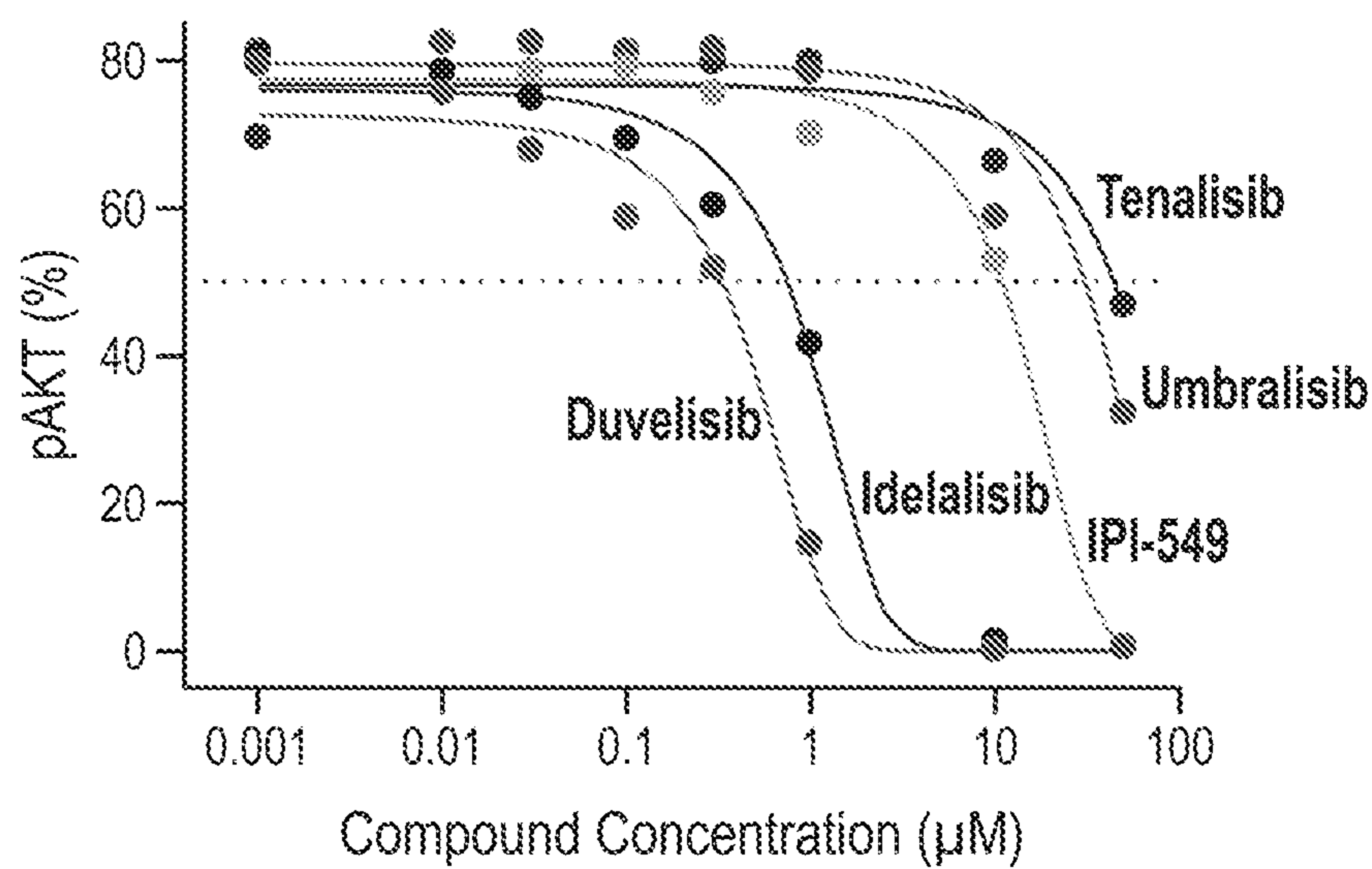
PI3K- δ Whole Blood Assay LPS-Stimulated Monocytes

FIG. 1

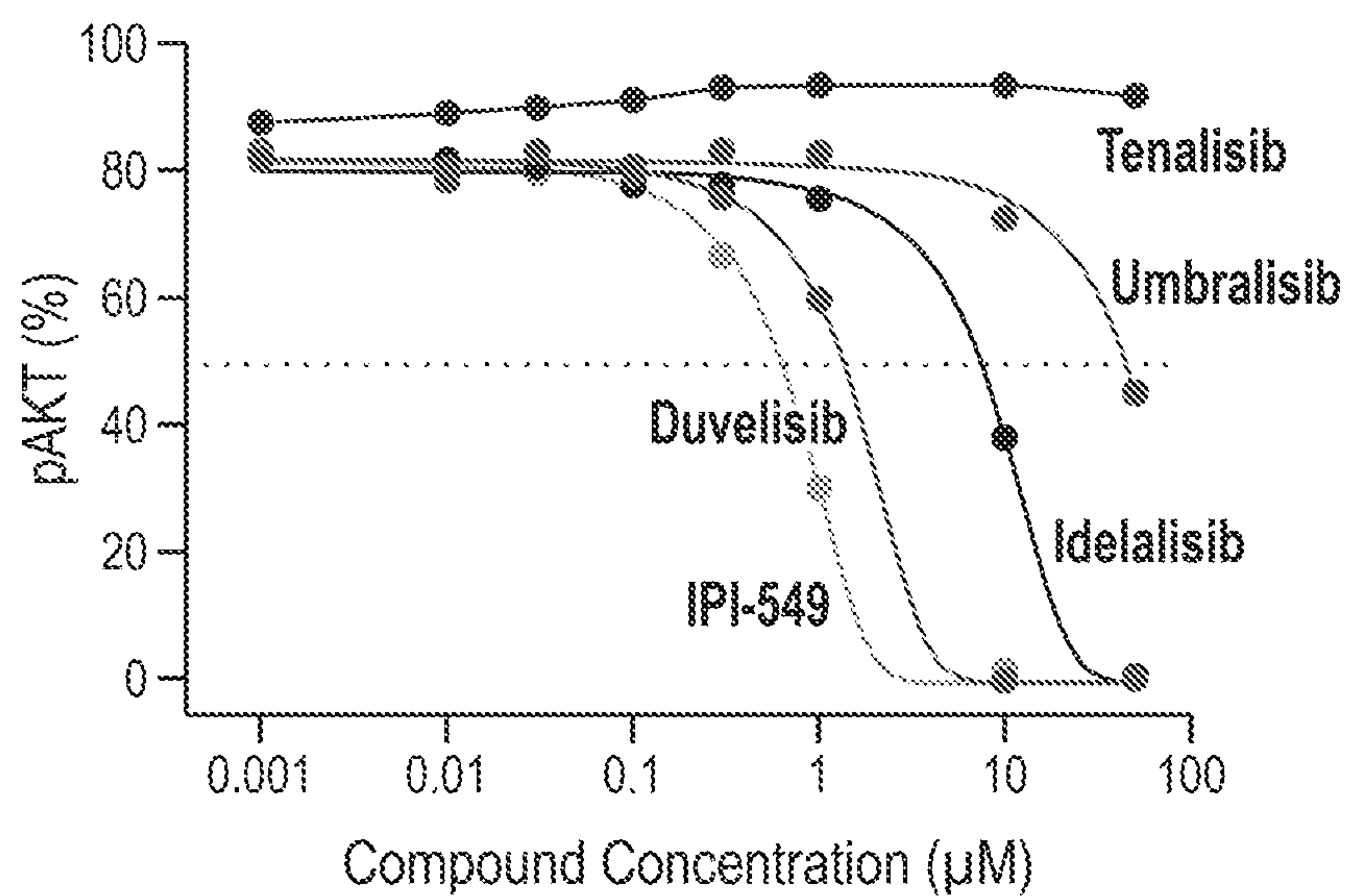
PI3K- γ Whole Blood Assay fMLP-Stimulated Monocytes

FIG. 2

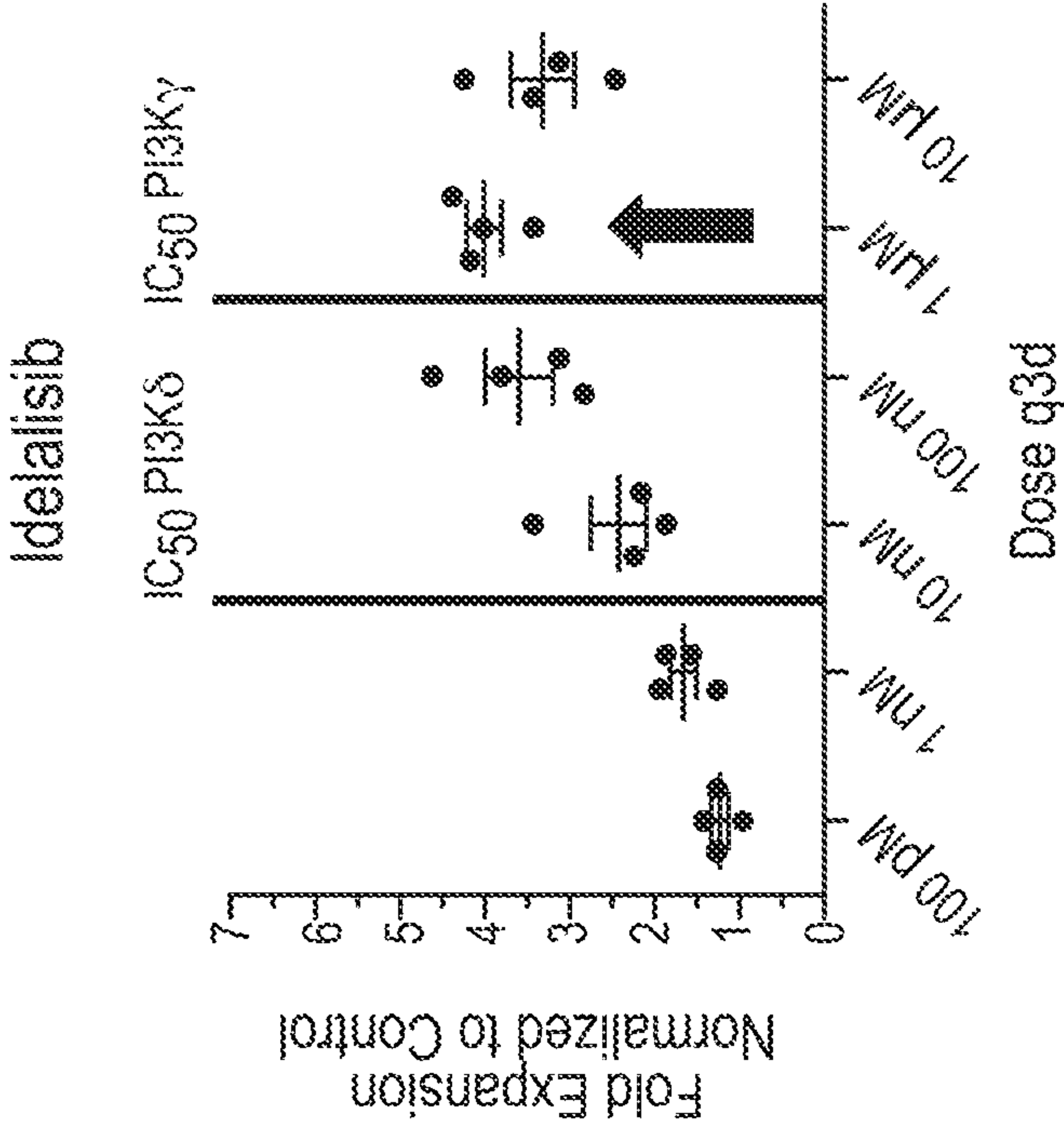


FIG. 3B

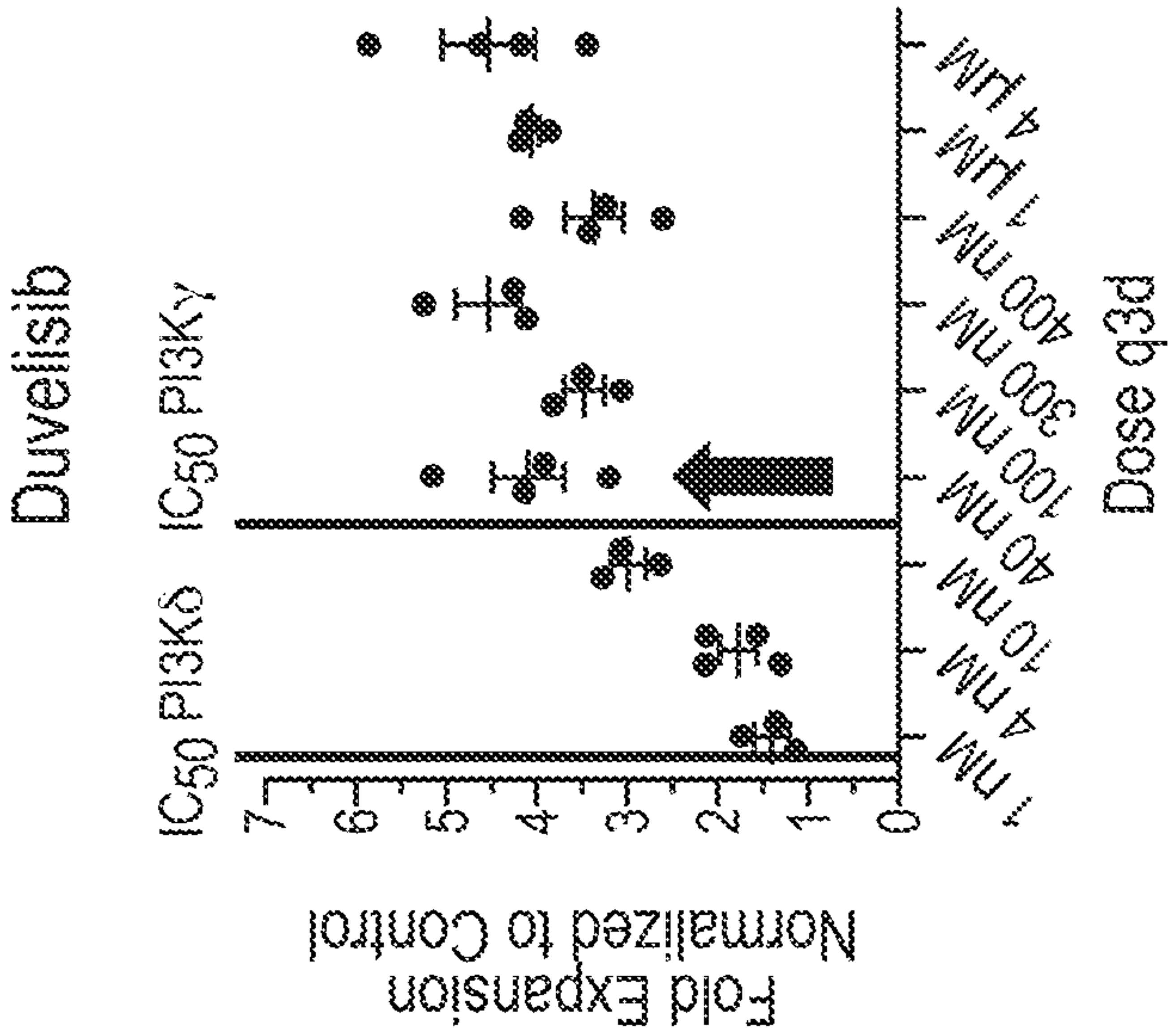


FIG. 3A

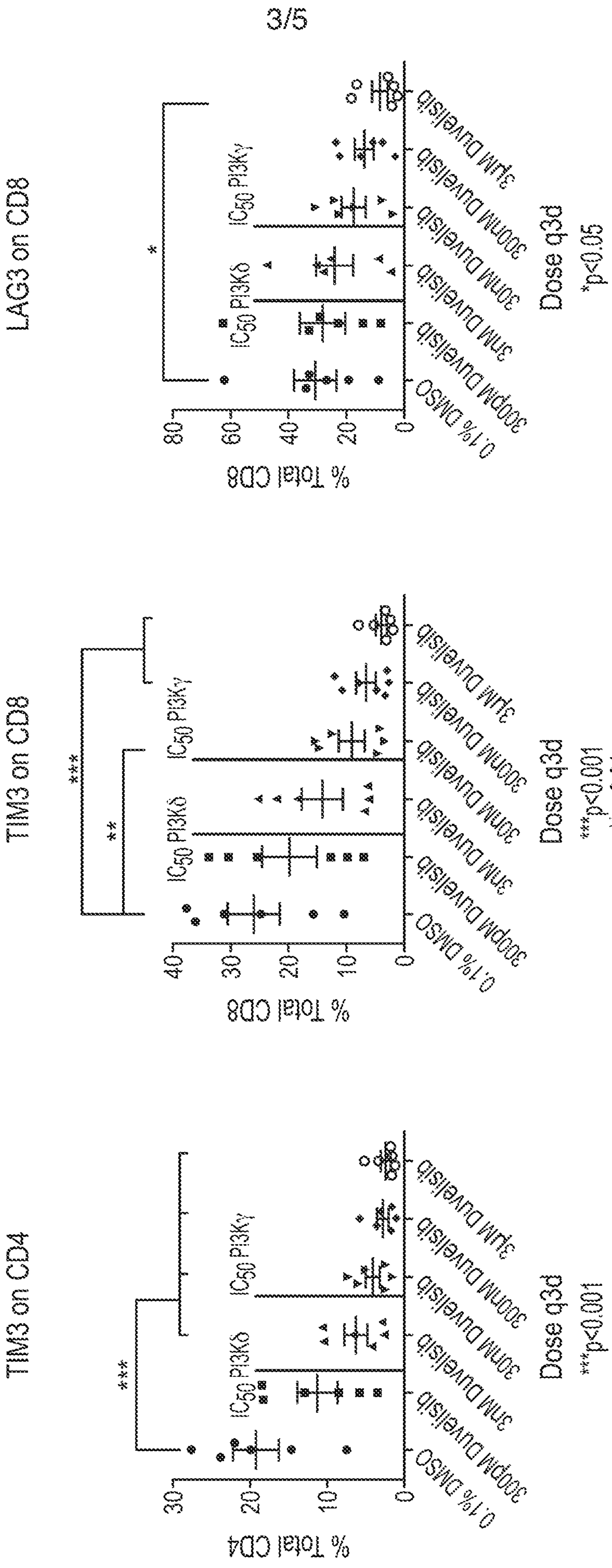


FIG. 4A

FIG. 4B

FIG. 4C

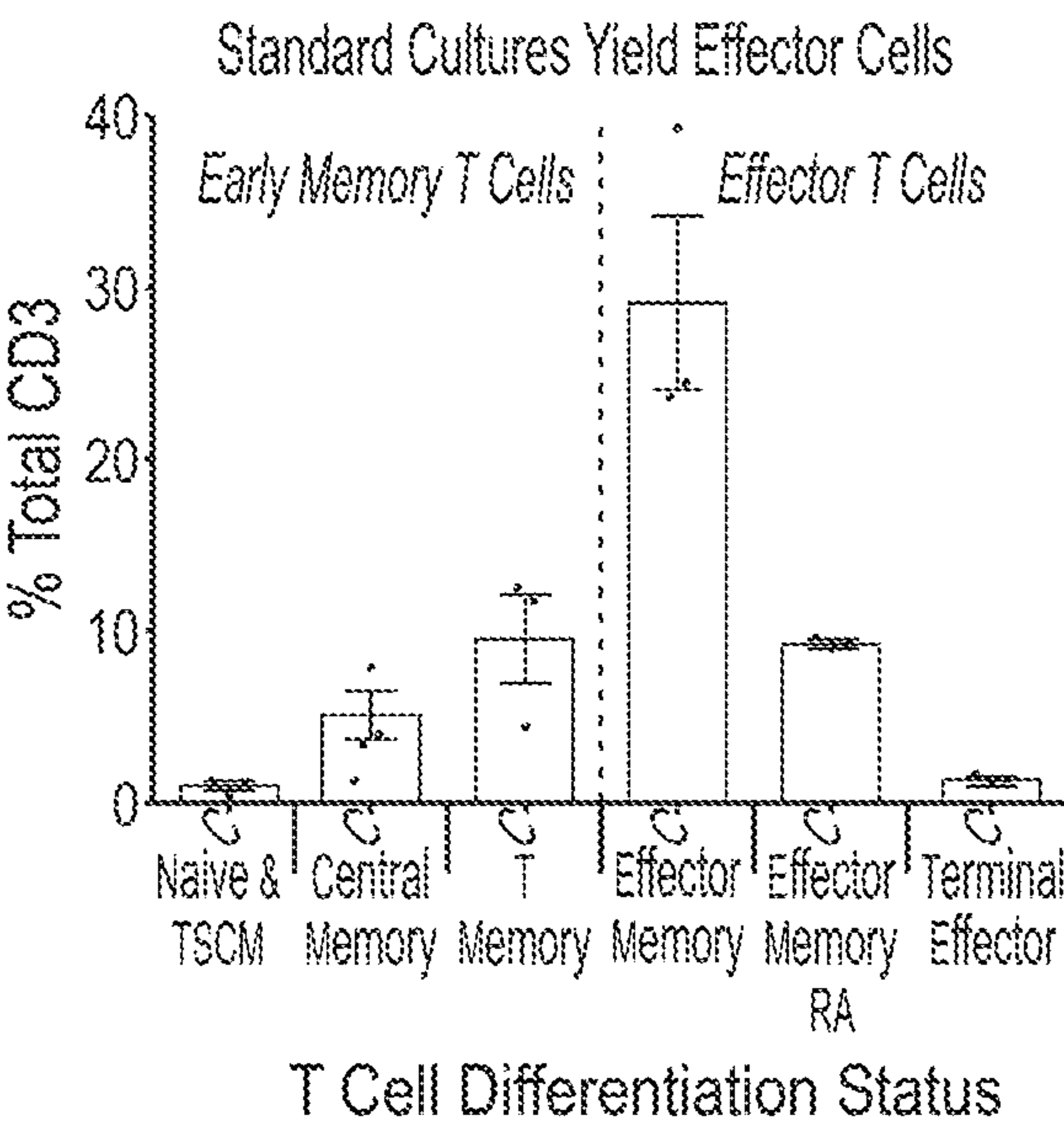


FIG. 5A

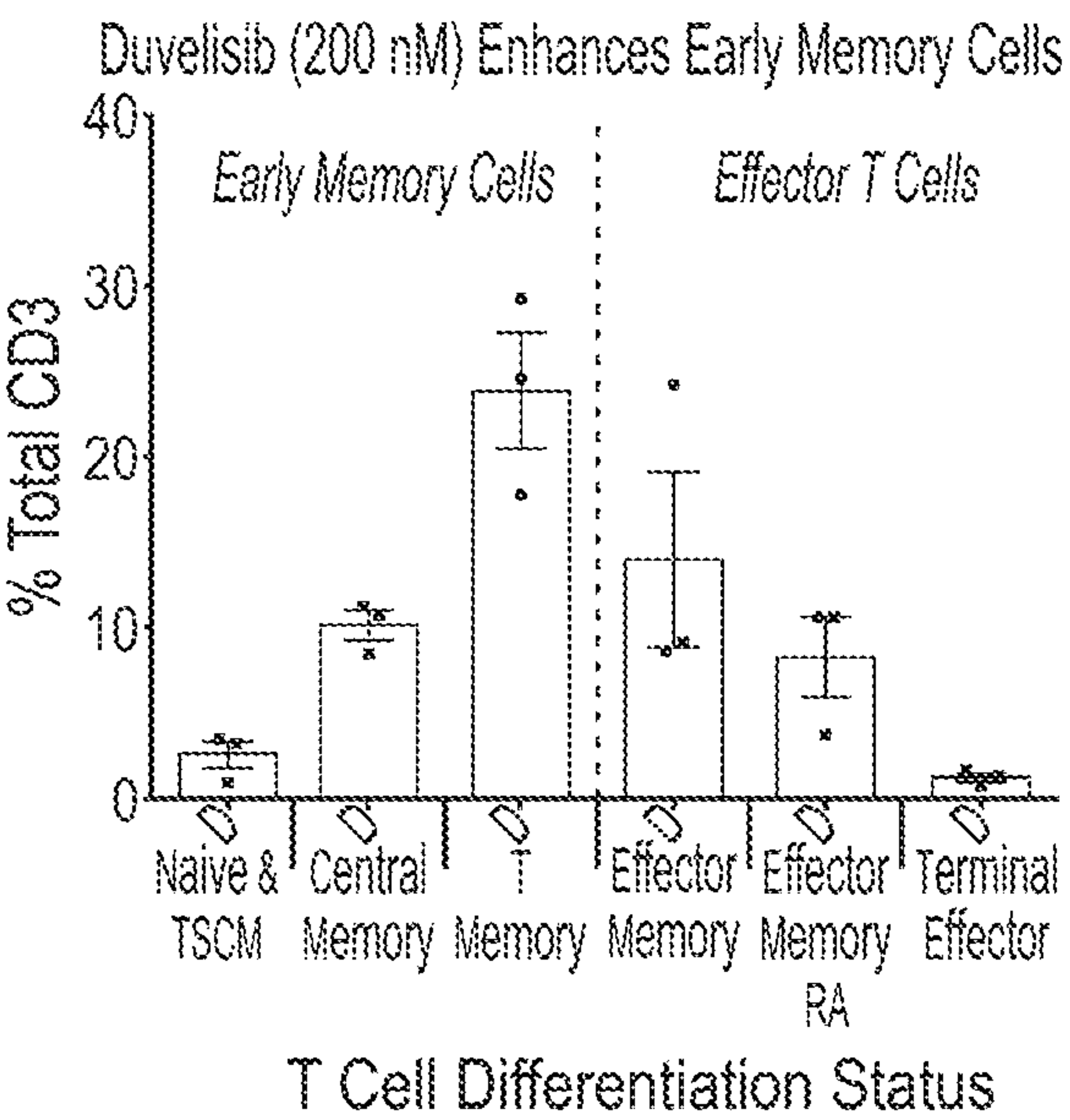


FIG. 5B

Duvelisib increases CD8:CD4 CAR T ratio

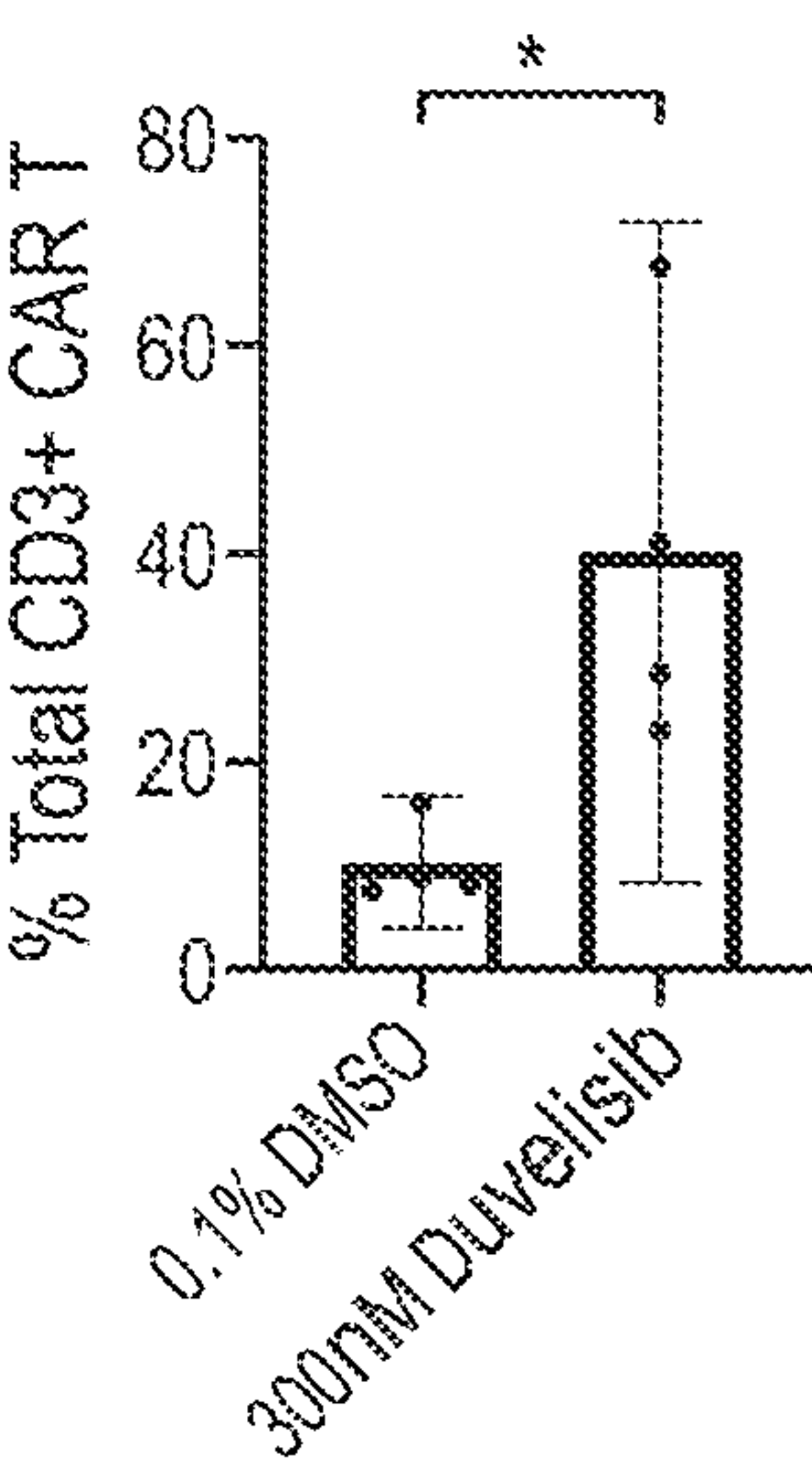


FIG. 6A

Duvelisib augments CAR T cytotoxicity

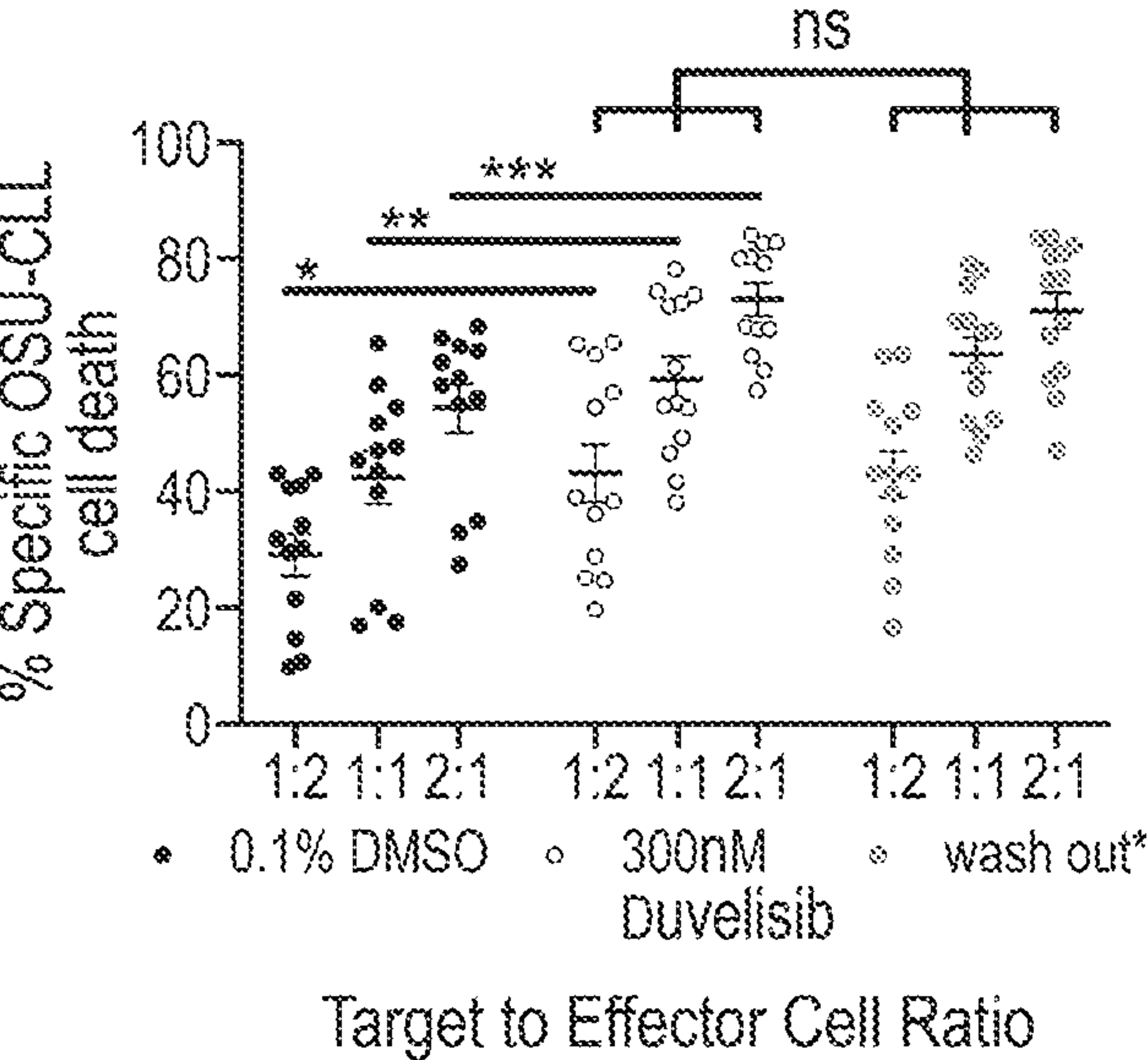


FIG. 6B

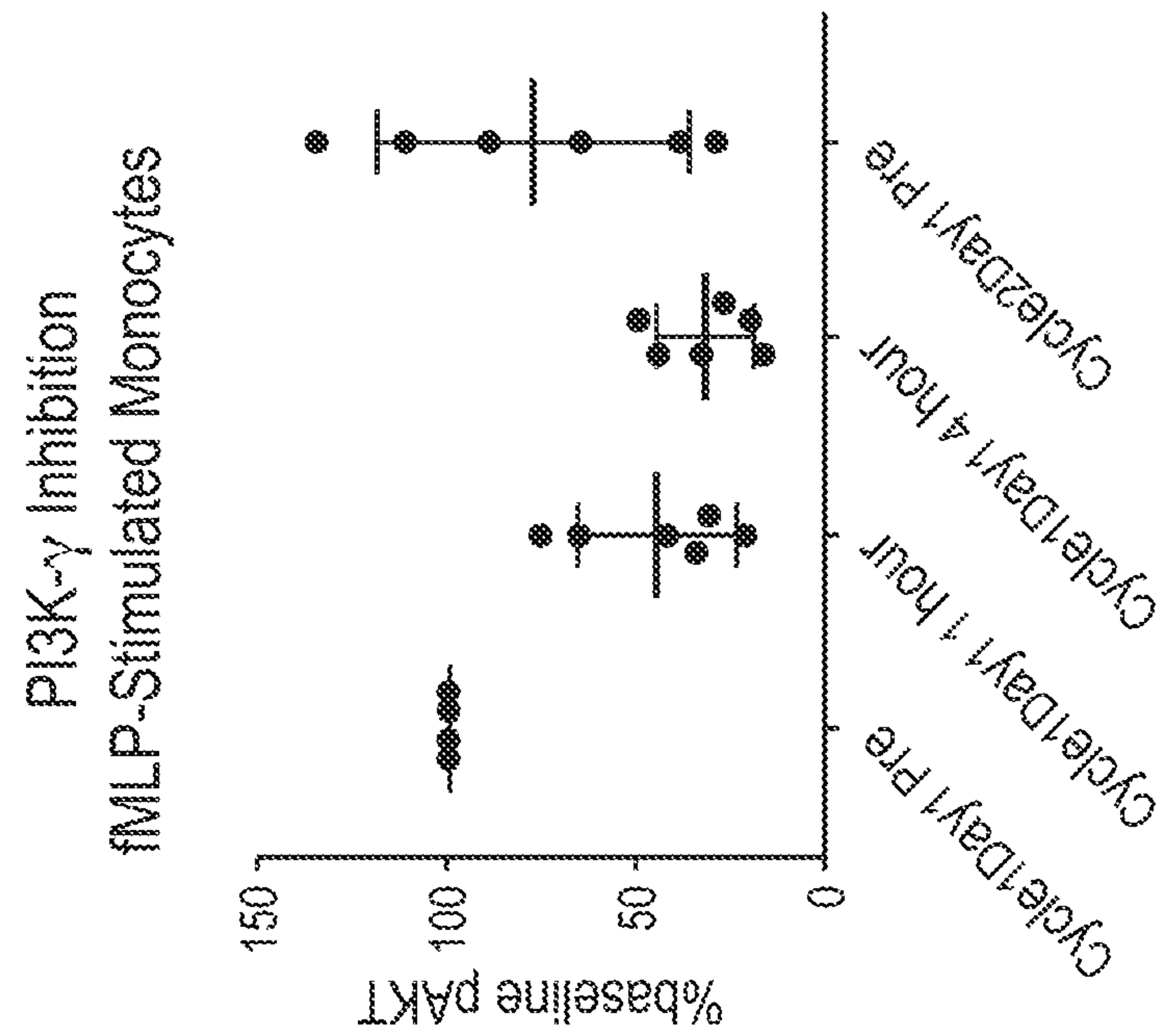


FIG. 7B

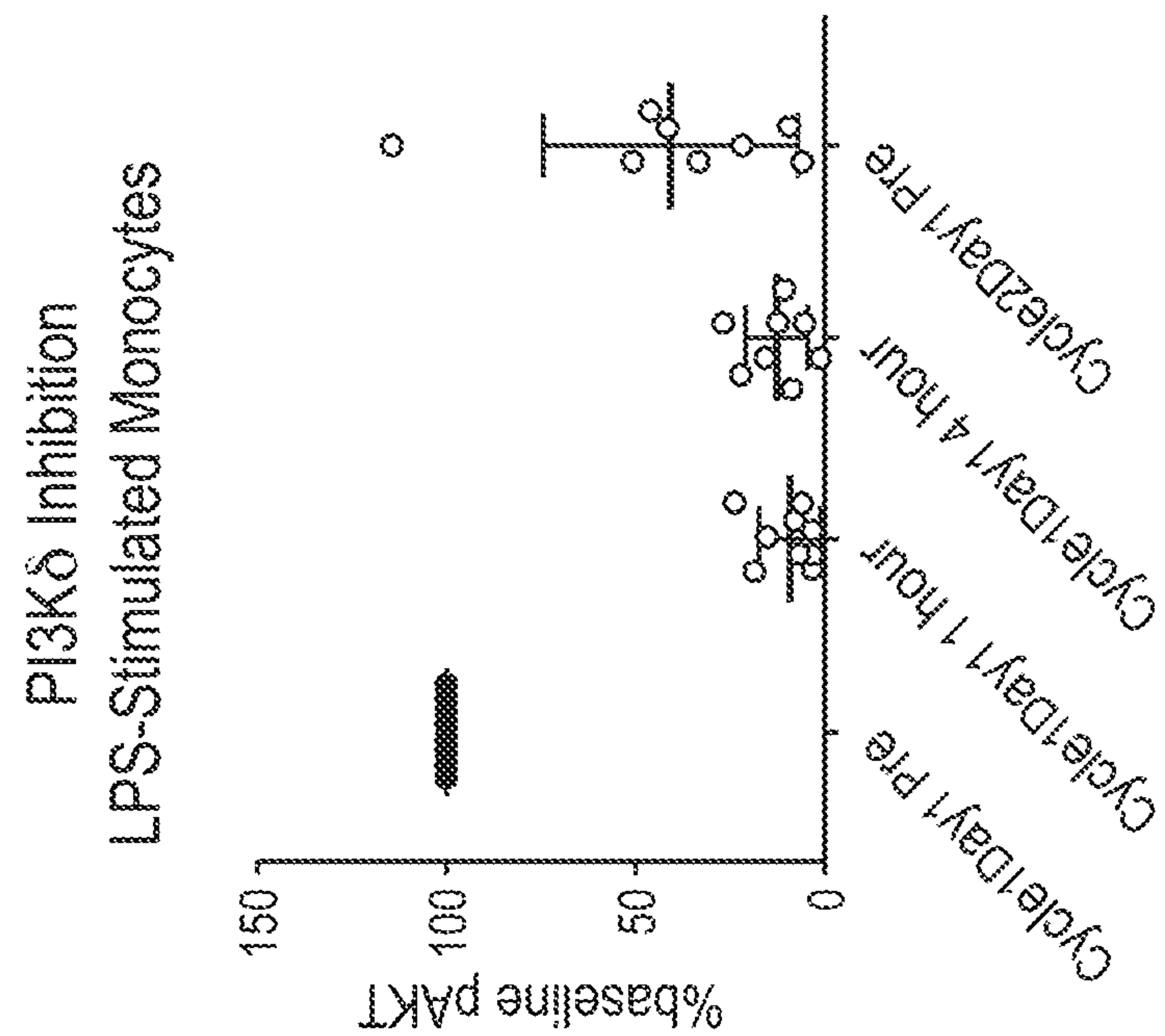


FIG. 7A

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/58870

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 35/17, C07K 16/28, C07K 14/725 (2020.01)

CPC - C12N 5/0636, A61K 35/17, C07K 2319/02, C07K 2319/03

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/110667 A1 (CELYAD S.A.) 13 June 2019 (13.06.2019) abstract, pg 19, ln 3-19, pg 22, ln 28 to pg 23, ln 4, pg 26, ln 12-14, pg 34, ln 3-9	1-3
X	US 2017/0335281 A1 (LOEW et al.) 23 November 2017 (23.11.2017) abstract, para [0902], [0947]	22-26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

07 January 2021

Date of mailing of the international search report

04 FEB 2021

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Lee Young

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 20/58870

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-21, 27-33
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.