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- (71) **Applicant: UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.** [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).
- (72) **Inventor: CHANG, Lung-Ji;** 3102 NW 57th Terrace, Gainesville, FL 32606-6939 (US).
- (74) **Agent: MACDONALD, Kevin;** Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).
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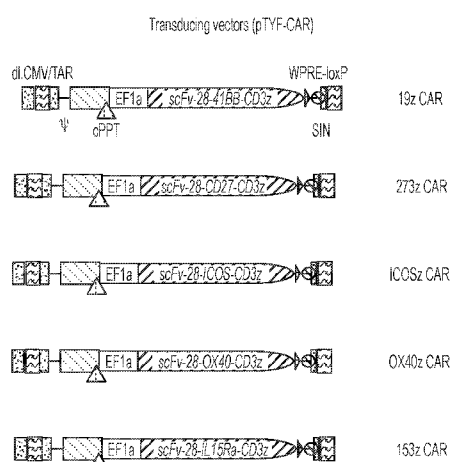
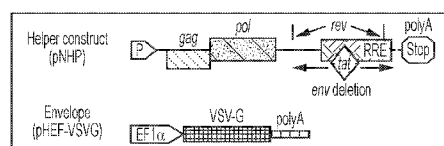
(54) **Title:** CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

FIG. 1

(57) **Abstract:** Provided herein are chimeric antigen receptors (CARs) comprising an antigen binding domain (e.g., CD19, CD30, GD2, etc.), transmembrane domain (e.g., CD28), and a cytoplasmic domain (e.g., CD27, 4-1BB, etc.). In some aspects, the disclosure relates to use of the CARs in T cells, compositions, kits and methods.



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CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

RELATED APPLICATIONS

This Application claims the benefit under 35 U.S.C. § 119(e) of U.S. provisional applications U.S.S.N. 62/118,080, filed on February 19, 2015, entitled “CHIMERIC ANTIGEN RECEPTOR COMPRISING INTERLEUKIN-15 RECEPTOR INTRACELLULAR DOMAIN AND USES THEREOF”, 62/134,444, filed on March 17, 2015, entitled “CHIMERIC ANTIGEN RECEPTORS THAT TARGET GD2”, 62/152,792, filed on April 24, 2015, entitled “CHIMERIC ANTIGEN RECEPTORS THAT TARGET CD30 AND USES THEREOF”, and 62/165,836, filed on May 22, 2015, entitled “CHIMERIC ANTIGEN RECEPTORS THAT TARGET GD2”, the entire content of each of which is incorporated by reference herein.

BACKGROUND

Chimeric Antigen Receptors (CARs) are engineered T cell receptors displaying specificity against target antigens based on a single chain FV (scFv) antibody moiety. Although CARs show promise as therapeutic agents, several challenges (*e.g.*, the induction of antigen-specific toxicities targeting normal tissues expressing the target-antigen, and the extreme potency of CAR-T cell treatments resulting in life-threatening cytokine-release syndromes) limit their use in a clinical context. Therefore, there is a need to develop safer and more clinically effective CARs.

SUMMARY

In some aspects, the disclosure relates to chimeric antigen receptors (CARs) comprising an antigen binding domain (*e.g.*, anti-CD19, anti-CD30, anti-GD2), and/or a cytoplasmic domain of a interleukin(IL)-15-receptor α , and/or a chimeric cytoplasmic domain including a CD27 cytoplasmic domain fused to a 4-1BB cytoplasmic domain. Use of the CARs in T cells, compositions, kits and methods is also contemplated by the disclosure.

Aspects of the disclosure relate to CARs comprising a cytoplasmic domain of a

interleukin(IL)-15-receptor α , and uses of such CARs to produce CAR T-cells (CARTs) and/or CAR-modified immune cells such as NK (natural killer) cells, which can be used in various methods, such as treatment methods, or compositions.

As described herein, it was found that including the cytoplasmic domain of IL-15-receptor α (IL-15R α) in a CAR construct resulted in CARTs with greater expansion potential upon antigen stimulation and higher killing efficacy. Further, the CARTs maintained killing efficacy even on repetitive addition of an excess of target cells. The CARTs also produced increased amounts of effector cytokines, as determined by intracellular staining and flow cytometry based bead assays.

Accordingly, aspects of the disclosure relate to a chimeric antigen receptor (CAR) comprising an antigen binding domain; a transmembrane domain; and a cytoplasmic domain containing an interleukin 15-receptor α (IL-15R α) cytoplasmic domain. In some embodiments, the transmembrane domain is a CD28 or CD8 transmembrane domain. In some embodiments, the cytoplasmic domain further comprises a CD3zeta signal transduction domain. In some embodiments, the cytoplasmic domain further comprises a CD27 signaling domain. In some embodiments, the antigen binding domain is a single-chain variable fragment (scFv). In some embodiments, the antigen binding domain is specific for CD19.

Aspects of the disclosure relate to CARs comprising a chimeric cytoplasmic domain including a CD27 cytoplasmic domain fused to a 4-1BB cytoplasmic domain, and uses of such CARs to produce CARTs and/or CAR-modified immune cells such as NK (natural killer) cells, which can be used in various methods, such as treatment methods, or compositions.

In some aspects, the disclosure relates to a chimeric antigen receptor (CAR) comprising: an antigen binding domain; a transmembrane domain; and a cytoplasmic domain containing a CD27 intracellular domain.

In some embodiments, the transmembrane domain is a CD28 or CD8 transmembrane domain. In some embodiments, the cytoplasmic domain further comprises a 4-1BB intracellular domain. In some embodiments, the cytoplasmic domain further comprises a CD3zeta signal transduction domain. In some embodiments, the cytoplasmic domain further

comprises an iCasp9 domain and/or a FKBP domain. In some embodiments, the antigen binding domain is a single-chain variable fragment (scFv).

In some embodiments, a CAR comprises a CD28 transmembrane domain, cytoplasmic domain comprising a CD27 intracellular domain and a 4-1BB intracellular domain, and a CD3zeta signal transduction domain. In some embodiments, a CAR further comprises an iCasp9 domain and/or a FKBP domain.

In some embodiments, a CAR further comprises a first spacer between the CD28 transmembrane domain and the cytoplasmic domain comprising a CD27 intracellular domain and a 4-1BB intracellular domain, and a second spacer between the cytoplasmic domain comprising a CD27 intracellular domain and a 4-1BB intracellular domain and the CD3zeta signal transduction domain. In some embodiments, the CAR further comprises a third spacer between the CD3zeta signal transduction domain and the iCasp9 domain and/or a FKBP domain. In some embodiments, the antigen binding domain of a CAR is specific for CD30.

Aspects of the disclosure relate to CARs comprising an antigen-binding domain specific for Disialoganglioside 2 (GD2), and uses of such CARs to produce CAR-modified immune cells such as T cells (also referred to herein as CARTs) or NK (natural killer) cells, which can be used in various methods, such as treatment methods, or compositions. In some aspects of the disclosure, such CARs are used to treat retinoblastoma or osteosarcoma, such as a juvenile subject having retinoblastoma or osteosarcoma.

As described herein, it was found that GD2-specific CAR-expressing T cells could kill both osteosarcoma and retinoblastoma cells, both of which express GD2. As a result, it is expected that GD2-specific CARs will be useful for treatment of both osteosarcoma and retinoblastoma.

Aspects of the disclosure relate to a chimeric antigen receptor (CAR) comprising an antigen binding domain specific for GD2; a transmembrane domain; and a cytoplasmic domain containing one or more (*e.g.*, one, two, or three) of a CD27 signaling domain, a 4-1BB intracellular domain, and a CD3zeta signal transduction domain. In some embodiments, the transmembrane domain is a CD28 or CD8 transmembrane domain. In some embodiments, the antigen binding domain is a single-chain variable fragment (scFv).

Other aspects of the disclosure relate to a nucleic acid comprising a sequence that encodes the CAR of any one of the above embodiments or as otherwise described herein.

Yet other aspects relate to an immune cell comprising a CAR of any one of the above embodiments or as otherwise described herein and/or a nucleic acid of any one of the above
5 embodiments or as otherwise described herein. In some embodiments, the immune cell is a T cell or NK cell. In some embodiments, the immune cell is a T cell.

Other aspects of the disclosure relate to a composition comprising a plurality of the immune cell of any one of the above embodiments or as otherwise described herein. In some
embodiments, the composition comprises a pharmaceutically acceptable carrier.

10 Another aspect of the disclosure relates to a method of generating a plurality of CAR-modified immune cells, the method comprising introducing a lentiviral vector comprising a nucleic acid of any one of the above embodiments or as otherwise described herein into a plurality of immune cells. In some embodiments, the immune cells are T cells.

Other aspects of the disclosure relate to a method of treating a subject having a
15 disease, the method comprising administering an immune cell of any one of the above embodiments or as otherwise described herein, the composition of any one of the above embodiments or as otherwise described herein, or the plurality of immune cells produced by a method of any one of the above embodiments or as otherwise described herein into a subject having a disease or at risk of having a disease.

20 In some embodiments, the disease is cancer, an autoimmune disease or an infection. In some embodiments, the disease is cancer. In some embodiments, the disease is a CD30+ cancer. In some embodiments, the cancer is osteosarcoma or retinoblastoma.

In some embodiments, the method further comprises administering a PD-L1 or PD1 inhibitor to the subject.

25 Other aspects of the disclosure relate to a method of treating a subject having cancer, the method comprising administering (a) an immune cell expressing a CAR that targets GD2 and (b) a PD-L1 or PD1 inhibitor to a subject having cancer. In some embodiments, the cancer is osteosarcoma or retinoblastoma. In some embodiments, the immune cell is a T cell. In some embodiments, the immune cell is a plurality of immune cells in a composition.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure, which can be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein. It should be appreciated that in greyscale versions of the drawings, GFP fluorescence appears as areas of lighter shading (*e.g.*, white shading)

FIG. 1 is a diagram showing an exemplary Chimeric Antigen Receptor (CAR) basic structure including the antigen binding scFv domain and the various co-stimulatory domains.

FIG. 2 is a diagram showing an exemplary simple killing assay- Co-culture of green fluorescent protein wasabi (GFP)-labeled target with the CART effector cells and flow cytometry after annexinV/PI staining.

FIG. 3 is a series of plots and two photographs showing that exemplary lentiviral gene transfer into human T cells is very high. The X-axis in each plot shows CD3 staining and the Y-axis in each plot shows GFP staining.

FIG. 4 is a series of photographs showing exemplary primary T cell killing efficiency demonstrated by disappearance of the green (fluorescent) target cells as seen under the fluorescent microscope. Both bright-field and fluorescent images are shown for each sample.

FIG. 5A is a series of plots and a graph showing exemplary healthy donor derived CART cells -Flow data of Killing in terms of % early apoptotic cells with annexinV positive and PI negative staining seen after 1-2 hours of co-culture.

FIG. 5B is two graphs showing exemplary % total cell death (top graph) for each CAR and % specific lysis (bottom graph) for each CAR. This was done in triplicate wells to acquire mean values and statistical significance obtained using student t test ($p < 0.05$ considered significant). * $P < 0.01$ (comparing with No CAR), # $P < 0.01$ (comparing with a control CART), ^ $P < 0.05$ (comparing 153z with 19z and 273z).

FIG. 6A is a series of graphs showing exemplary flow data of killing after overnight incubation in terms of % early and late apoptotic cells (both annexinV + PI- and double

positive for AnnexinV and PI)

FIG. 6B is a series of plots and graphs showing exemplary long term killing after 3 days of co-coculture of patient derived CART cells with green (fluorescent) target cells. Killing was indicated by disappearance of the green target cells.

5 **FIG. 6C** is a series of photographs and a graph showing exemplary long term killing after 3 days of co-coculture of patient derived CART cells with green (fluorescent) target cells. Killing was indicated by disappearance of the green (fluorescent) target cells.

FIGs. 7A and 7B show a series of plots and graphs of exemplary patient ALL cells labeled with Calcein AM and patient derived CART cells incubated in 5:1 ratio. Flow data 10 shows killing at 1 hour (FIG. 7A) and 1 day (FIG. 7B) in terms of early apoptosis with % annexinV positive and PI positive cells.

FIG. 8 is a series of plots showing exemplary retargeting of CART effector cells with green (fluorescent) target. Killing seen as indicated by disappearance of target in flow cytometry.

15 **FIG. 9** is a series of plots and a graph showing exemplary CART cell proliferation by a CFSE method. Proliferation peak and % seen after CART cells stimulation with target cells.

FIGs. 10A and 10B are a series of graphs showing exemplary intracellular effector function analysis flow data of IL-2, IFN-g and CD107a produced after the CART cells are 20 co-cultured with the target cells- RS4-11. Positive control (PC) is PMA/ionomycin stimulation.

FIG. 10C is a graph showing the exemplary data summarized from FIGs. 10A and 10B in bar graph form. Maximum IL-2, IFN-g and CD107a release was seen with 153z CART cells.

25 **FIG. 11** shows flow cytometry analysis of CD30 surface expression of a primary B cell tumor line.

FIG. 12 shows an illustration of the lentiviral vector system.

FIG. 13 shows diagrams illustrating embodiments of CAR molecules, including one embodiment of a 4th generation CD30-CAR structure and components.

FIG. 14 shows an illustration of a rapid CAR T target killing assay used for the evaluation of CAR functions.

FIG. 15 shows data related to a target killing assay of two different CD30-CAR T cells based on 5F11 and AC10 scFv CD30-CARs.

5 **FIG. 16** shows flow cytometry target killing assay of two different CD30-CAR T cells based on 5F11 and AC10 scFv CD30-CARs.

FIG. 17 shows the clinical course of a Hodgkin's lymphoma patient treated with CD30-CAR T cells. The infusion time point was depicted by arrows. Three batches of CAR T cells were prepared and infused as indicated. CAR-positive T cells were detected by qPCR
10 detection of CAR DNA in the genome of blood cells. Clinical evidence of tumor regression was evident at day 40 after the first infusion.

FIG. 18 shows data related to detection of IL-6 cytokine response after CD30 CAR T infusion.

FIG. 19 shows data related to detection of IFN-gamma cytokine response after CD30
15 CAR T infusion.

FIGs. 20A-20C shows graphs of exemplary GD2 surface expression in osteosarcoma (OS) cell lines. Flow cytometry histogram overlays show surface expression of GD2 in (**FIG. 20A**) HOS (**FIG. 20B**) U2OS and (**FIG. 20C**) OS156 cells. The dark line represents GD2 positive and the shadowed grey line represents isotype control. Inner number indicates
20 percent positive population and mean fluorescence intensity (MFI) of the positive population

FIG. 21A is a schematic showing an exemplary CAR that targets GD2 binding to a cell expressing GD2.

FIG. 21B is a schematic that shows embodiments of CAR molecules, including one embodiment of GD2 CAR construction and 4th generation CAR configurations.

25 **FIG. 22** is a diagram that shows an exemplary CAR-JK/T cell killing assay.

FIGs. 23A-23D are a series of plots and graphs that show short term GD2 JK killing. JK cells were used for CAR construction as a preliminary determination of GD2 CAR killing ability. **FIG. 23A**) Representative dot plots from short term killing of U2OS cells by GD2 CAR-JK cells (E:T ratio =2:1). Effector and target cells are separated by FITC signal, FITC

positive target cells (upper panels) were gated and determined percentage of death cell by Annexin V versus PI plots (lower panels). **FIG. 23B)** Percent specific lysis of U2OS by GD2 CAR-JK cell was presented in comparison with control CAR-JK cell, * indicates a significant difference ($p = 0.0049$). **FIG. 23C)** Representative dot plots from short term killing of HOS cells by GD2 CAR-JK cells (E:T ratio =2:1) showing percentage of FITC positive target cells (upper panels) and Annexin V and/or PI positive target cells (lower panels). **FIG. 23D)** Percent specific lysis of HOS by GD2 CAR-JK cell shows higher but not significant different compare to control CAR-JK cells ($p = 0.078$; NS = not significant different).

FIGs. 24A-24G show exemplary CAR-Primary T cell killing of OS target. The killing ability of GD2 CAR in primary T cells were determined. **FIG. 24A)** Flow cytometry analysis of U2OSw cells by GD2 CAR-T cells after co-culture at E:T ratio of 4:1, 2:1, 1:1, 1:2, * indicates significant different from controls ($p < 0.05$). **FIG. 24B)** Percent specific lyses of U2OSw cells after 3 day co-culture, * indicates significant different from control T cells ($p < 0.05$). **FIG. 24C)** Flow cytometry analysis of HOSw cells by GD2 CAR-T cells after co-culture at E:T ratio of 4:1, 2:1, 1:1, 1:2, * indicates significant different from controls ($p < 0.05$). **FIG. 24D)** Percent specific lyses of HOSw cells after 3 day co-culture, * indicates significant different from control T cells ($p < 0.05$). **FIG. 24E)** Percent specific lyses of OS156 cells after 3 day co-culture, * indicates significant different from control T cells ($p < 0.05$). **FIG. 24F)** Target cells (green, fluorescent) and effector cells (no color) under fluorescence microscope after 4 days co-culture. **FIG. 24G)** Target cells (green, fluorescent) and effector cells (no color) under fluorescence microscope after 3, 7 or 12 days co-culture.

FIGs. 25A-25D show exemplary PDL-1 expression in OS cells after incubation with CAR T cells. **FIG. 25A)** and **FIG. 25B)** Flow cytometry histogram overlays show surface expression of PD-L1 in U2OS and HOS cell. The shadowed area represents isotype control. Inner number indicates percent positive population and MFI of the positive population. **FIG. 25C)** and **FIG. 25D)** MFI analysis of PD-L1 expression in OS cells after CAR T co-culture.

FIGs. 26A-26F show exemplary PD-1 expression in CAR T cells after incubation with OS cells. PD1 expression in primary CAR T cells after 1 day co-culture with target cells

(E:T ratio = 2:1), U2OSw (**FIGs. 26A-26B**) and HOSw (**FIGs. 26D-26E**). A bar graph displays fold increase of PD1 expression from background expression level. The number above the bars show fold increase of PD1 expression. **FIG. 26C**) and **FIG. 26F**) Increased CAR T cell death after PD-1 up-regulation. Percent CAR T cell death after 1 day co-culture with U2OSw (C) and HOSw (D) cells, # indicates significant different at $p < 0.05$.

FIG. 27A is a series of photographs that shows immunohistochemistry analyses of GD2 expression in tumor samples from RB patients. Paraffin-embedded retinal tumor samples were stained for GD2 expression by using antibody against human GD2 protein.

FIG. 27B is a series of graphs that shows surface staining of GD2 expression on Y79 retinoblastoma (RB) cell line. Flow cytometric staining for GD2 and CD19 proteins (black line) or an IgG control (filled histogram) on the surface of Y79 RB cells. Upper right number indicates cell count and the normalized mean fluorescence intensities (nMFI) are shown in parenthesis.

FIG. 28A is a diagram of exemplary GD2 CAR constructs.

FIG. 28B is a diagram showing an exemplary cell killing assay for RB cells. Y79 RB cells were engineered to express wasort, a green fluorescent protein (GFP), using lentiviral transduction. Y79 RB wasort cells were co-cultured with control T cells or CD19 T cells, or GD2 CAR T cells. After 24 hr of co-cultivation, apoptosis of tumor cells were examined by using AnnexinV/PI staining and analyzed by flow cytometry.

FIGs. 29A-29D show an exemplary short-term killing assay on Y79 RB using CAR T-cells. **FIG. 29A** shows analysis of cell death by using AnnexinV/PI staining. After 24 hr of co-culture experiment, the suspension cells were harvested and stained with AnnexinV-PE/PI dye followed by flow cytometric analysis. **FIG. 29B** shows the percent cell death at 1 day after co-culture. **FIG. 29C** shows the % of specific lysis. **FIG. 29D** is a series of photographs showing fluorescence microscopic monitoring of tumor cell lysis. Y79 RB wasort cells (1×10^5) were cultured with control T cells or CD19 CAR T cells, or GD2 CAR T cells at the effector/target ratio (1:1) for 24 h, and the lysis of tumor cells were monitored under fluorescence microscope.

FIG. 30 is a series of photographs that shows exemplary 2nd round killing of target

RB cells by GD2-CAR T cells at day 6 or day 14 of co-culture.

FIG. 31A and 31B are a series of graphs that show down-regulation of GD2 in RB tumor upon GD2-CAR T targeting.

FIG. 31C is a series of graphs that shows upregulation of PD-L1 expression upon
5 GD2-CAR T cell targeting.

FIG. 31D is a series of graphs that shows down-regulation of GD2 expression upon GD2-CAR T cell targeting.

DETAILED DESCRIPTION

10 In some aspects, the disclosure relates to chimeric antigen receptors (CARs) comprising an antigen binding domain (*e.g.*, anti-CD19, anti-CD30, anti-GD2), and/or a cytoplasmic domain of a interleukin(IL)-15-receptor α , and/or a chimeric cytoplasmic domain including a CD27 cytoplasmic domain fused to a 4-1BB cytoplasmic domain. Use of the CARs in T cells, compositions, kits and methods is also contemplated by the disclosure.

15 The invention relates, in some embodiments, to chimeric antigen receptors (CARs) and uses thereof in T cells (*e.g.*, to make CAR T cells), methods, nucleic acids, compositions, kits and the like. CARs are molecules that combine antibody-based specificity for a desired antigen (*e.g.*, tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-tumor cellular immune activity. It should be
20 appreciated that, in some embodiments, exemplary, non-limiting arrangements of CARs are described from left to right, N-terminus to C-terminus of the CAR.

In some aspects, the disclosure relates to CARs having an interleukin 15-receptor α (IL-15R α) cytoplasmic domain. CARs containing this domain had several advantageous and surprising features including greater expansion potential upon antigen stimulation, higher
25 killing efficacy, maintained killing efficacy upon repetitive addition of excess target cells, and an increased amount of effector cytokines compared to CARs not containing this domain.

In some embodiments, the present invention relates, in part, to the use of T cells genetically modified to stably express a desired CAR, *e.g.*, containing a IL-15R α cytoplasmic domain, containing a CD30 antigen binding domain, containing a GD2 antigen binding

domain, *etc.* T cells expressing a CAR are referred to herein as CAR T cells, CARTs, or CAR modified T cells. Preferably, the cell can be genetically modified to stably express an antibody binding domain on its surface, conferring novel antigen specificity that is MHC independent. In some instances, the T cell is genetically modified to stably express a CAR
 5 that combines an antigen recognition domain of a specific antibody with an transmembrane domain and a cytoplasmic domain into a single chimeric protein. In some embodiments, two CAR proteins dimerize (*e.g.*, form homo- or heterodimers) *in vivo*.

In some embodiments, a CAR comprises an antigen binding domain, a transmembrane domain, a cytoplasmic domain comprising an IL-15R α cytoplasmic domain,
 10 optionally further comprising a CD3 zeta (CD3z) signaling domain and/or a CD27 signaling domain. In some embodiments, the arrangement of the elements of the CAR is selected from one of the following exemplary, non-limiting arrangements (from N-terminus to C-terminus):

scFv-CD28- IL-15R α -CD3z

scFv-CD28-CD27-IL-15R α -CD3z

15 scFv-CD28-(4-1BB)-CD27-IL-15R α -CD3z

scFv-CD8-CD27-IL-15R α -CD3z

scFv-CD8-(4-1BB)-CD27-IL-15R α -CD3z

In some aspects, the disclosure relates to CARs having a CD30 antigen binding domain and an intracellular domain comprising a 4-1BB intracellular domain and a CD27
 20 intracellular domain. In some embodiments, the CARs further comprise a self-destructive domain (*e.g.*, an iCasp9-FKBP domain) that further improves safety. CARs containing these domains had several surprising features including an acceptable safety profile, and mediate effective killing of CD30⁺ cancer cells both *in vitro* and *in vivo*.

In some embodiments, a CAR (*e.g.*, CAR having a CD30 antigen binding domain)
 25 comprises an antigen binding domain, a transmembrane domain, a cytoplasmic domain comprising a 4-1BB intracellular domain and a CD27 intracellular domain, optionally further comprising a CD3 zeta (CD3z) signaling domain, and/or an apoptosis-inducing iCasp9-FKBP domain. In some embodiments, the arrangement of the elements of the CAR is selected from one of the following exemplary, non-limiting arrangements (from N-terminus

to C-terminus):

scFv-CD28-(4-1BB)-CD27- CD3z-iCasp9-FKBP

scFv-CD28-CD27-(4-1BB)-CD3z-iCasp9-FKBP

scFv-CD8-(4-1BB)-CD27- CD3z-iCasp9-FKBP

5 scFv-CD8- CD27-(4-1BB)-CD3z-iCasp9-FKBP

In some aspects, the disclosure relates to the discovery that GD2 was expressed on both osteosarcoma and retinoblastoma cells and that GD2-specific CAR-expressing T cells could kill both osteosarcoma and retinoblastoma cells. As a result, it is expected that such CARs (*e.g.*, GD2-specific CARs) will be useful for treatment of both osteosarcoma and
 10 retinoblastoma. In some embodiments, GD2-specific CARs contain an antigen binding domain (such as an scFV) specific for GD2. In some embodiments, the arrangement of the elements of the CAR (*e.g.*, GD2-specific CAR) is selected from one of the following exemplary, non-limiting arrangements (from N-terminus to C-terminus):

GD2scFv-CD28-(4-1BB)-CD27-CD3z

15 GD2scFv-CD8-CD28-CD3z

GD2scFv-CD28-(4-1BB)-CD27-CD3z-Casp9-FKBP

GD2scFv-CD8-CD28-CD3z -Casp9-FKBP

A CAR molecule may also include several hinge elements and/or spacer sequences (such as between individual domain elements). In some embodiments, the spacer and/or hinge

20 sequences of the CAR are selected from one or more of the following exemplary sequences: GGGGS (SEQ ID NO: 1), GGGGSGGGGS (SEQ ID NO: 2), (GGGGS)_{x3} (SEQ ID NO: 3), GSTSGGGSGGGSGGGGSS (SEQ ID NO: 4), GSTSGSGKPGSSEGSTKG (SEQ ID NO: 5), GGGGSGGG (SEQ ID NO: 6), VEPKSCDKTHTCPPCP (SEQ ID NO: 7), LDPKSSDKTHTCPPCP (SEQ ID NO: 8), VEPKSPDKTHTCPPCP (SEQ ID NO: 9), or
 25 LDKTHTCPPCP (SEQ ID NO: 10).

In some embodiments, the present invention relates, in part, to a CAR that incorporates a series of domains that provide different functional aspects that may synergistically work together to improve efficacy. For example, the CAR may include one or more of: an antigen binding domain, a hinge domain, an antigen co-signaling domain that

stimulates activity (*e.g.*, an IL-15R α cytoplasmic domain, a 4-1BB cytoplasmic domain, or another cytoplasmic domain), a survival domain that increases T-cell or immune effector cell survival, a T-cell or immune effector cell memory domain, and an effector activating domain. Alternatively, the CAR may further include a domain that induces safety (*e.g.*, an apoptosis-inducing iCasp9-FKBP domain).

In some embodiments, a CAR of the invention comprises an extracellular domain having an antigen binding domain, a transmembrane domain, and a multi-functional cytoplasmic domain. In some embodiments, the CAR comprises a fully human antibody or antibody fragment. In some embodiments, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. In another embodiment, 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 to minimize interactions with other members of the receptor complex.

In some embodiments, the CAR T cells or other CAR-modified immune cells (*e.g.*, CAR-modified NK cells) of the invention are generated by introducing a lentiviral vector comprising a nucleic acid that encodes a desired CAR into T cells or other immune cells (*e.g.*, into NK cells). In some embodiments, the lentiviral vector comprises a nucleic acid that encodes a CAR comprising an antigen binding domain (*e.g.*, that targets CD19), a transmembrane domain, and a cytoplasmic domain. In some embodiments, the CAR T cells or other CAR-modified immune cells (*e.g.*, NK cells) of the invention are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control. In some embodiments, the CAR T cell or other CAR-modified immune cells of the invention can be generated by transfecting a transposon or RNA encoding the desired CAR, into the T cells or other immune cells. In some embodiments, the CAR is transiently expressed in the genetically modified CAR T cell or other CAR-modified immune cells.

In some embodiments, the invention relates to administering a genetically modified immune cell (*e.g.*, a genetically modified T cell or NK cell) expressing a CAR for the treatment of a patient having cancer or at risk of having cancer, or having an autoimmune disease or at risk of having an autoimmune disease, *e.g.*, using lymphocyte infusion. In some

embodiments, autologous lymphocyte infusion is used in the treatment. In some embodiments, autologous PBMCs are collected from a patient in need of treatment and immune cells (*e.g.*, T cells or NK cells) are activated and expanded using the methods described herein and known in the art and then infused back into the patient.

5 In some embodiments, the cancer is a CD30+ cancer (*e.g.*, a cancer expressing CD30). Examples of cancers expressing CD30 include Hodgkin's lymphoma (HL), anaplastic large cell lymphoma (ALCL), diffuse large B cell lymphoma, primary effusion lymphoma, adult T-cell leukemia/lymphoma, mycosis fungoides, extranodal natural killer/T-cell lymphoma and peripheral T/NK cell lymphoma. In some embodiments the cancer is a
10 GD2-expressing cancer, such as retinoblastoma or osteosarcoma.

Exemplary Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention
15 pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

"Activation", as used herein, refers to the state of a T cell that has been sufficiently
20 stimulated to induce detectable cellular proliferation. Activation can also be associated with induced cytokine production, and detectable effector functions. The term "activated T cells" refers to, among other things, T cells that are undergoing cell division.

The term "antibody," as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from
25 natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)₂, as well as single chain antibodies, human antibodies, and humanized antibodies (Harlow et al., 1999, In: Using

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

The term "antibody fragment" refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', F(ab')₂, and Fv fragments, linear antibodies, scFv antibodies, and multispecific antibodies formed from antibody fragments.

An "antibody heavy chain," as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

An "antibody light chain," as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations, κ and λ light chains refer to the two major antibody light chain isotypes.

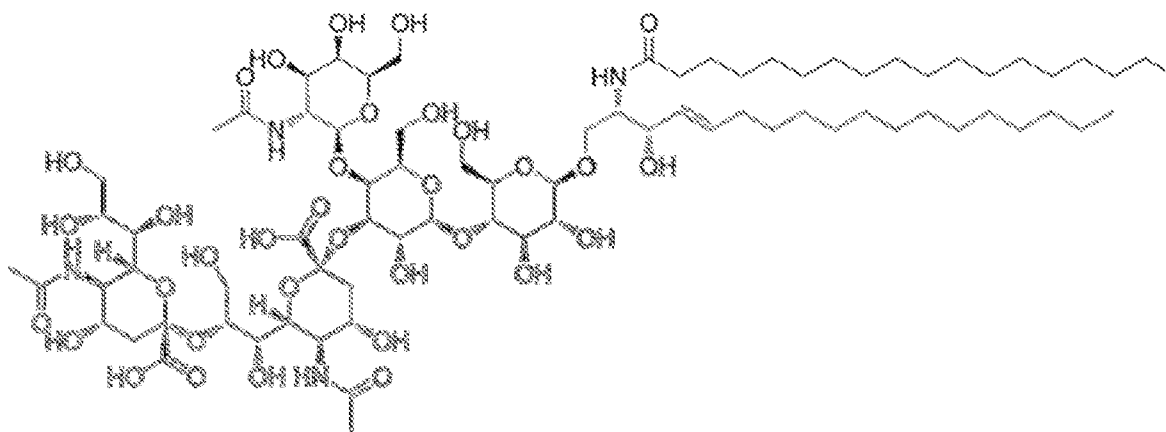
By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis 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 synthetic DNA or amino acid sequence technology which is available and well known in the art.

The term "antigen" or "Ag" as used herein is defined as 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. 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 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 elicit the desired immune response. Moreover, a skilled
 5 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 sample. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

The term "tumor antigen" as used herein refers to an antigen associated with a cancer
 10 cell. In some embodiments the tumor antigen is CD19, which is associated with B cell malignancies. In some embodiments, the tumor antigen is CD30, which is typically associated with lymphomas (*e.g.*, HL, ALCL, diffuse large cell B-lymphoma, *etc.* Examples of other tumor antigens include, but are not limited to, CD2, CD5, CD10, CD20, CD22, CD30, CD33, CD38, CD52, CD56, CD74, CD138, CD317, Her2, VEGFR2, EGFRviii,
 15 CXCR4, BCMA, GD2, GD3, and any other antigens over-expressed in tumor cells.

In some embodiments the tumor antigen is GD2. GD2 is a disialoganglioside that is expressed on cancer cells, such as retinoblastoma or osteosarcoma. An exemplary structure of GD2 is as follows:



20 The IUPAC name for GD2 is (2R,4R,5S,6S)-2-[3-[(2S,3S,4R,6S)-6-[(2S,3R,4R,5S,6R)-5-[(2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-2-[(2R,3S,4R,5R,6R)-4,5-dihydroxy-2-(hydroxymethyl)-6-[(E)-3-hydroxy-2-

(octadecanoylamino)octadec-4-enoyloxy]oxan-3-yl]oxy-3-hydroxy-6-(hydroxymethyl)oxan-4-yl]oxy-3-amino-6-carboxy-4-hydroxyoxan-2-yl]-2,3-dihydroxypropoxy]-5-amino-4-hydroxy-6-(1,2,3-trihydroxypropyl)oxane-2-carboxylic acid (see, *e.g.*, PubChem ID 6450346).

The term "target antigen" as used herein refers to an antigen associated with a disease-associated target cell. Examples of target antigens include but are not limited to CD2, CD5, CD7, CD10, CD19, CD20, CD22, CD30, CD33, CD38, CD52, CD56, CD74, CD138, CD317, Her2, VEGFR2, EGFRviii, CXCR4, BCMA, GD2, GD3, and any other antigens over-expressed in target cells or diseased cells. In some embodiments, the diseased cell is a B cell that produces auto-antibodies.

The term "anti-tumor effect" as used herein, refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An "anti-tumor effect" can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies of the invention in prevention of the occurrence of tumor in the first place.

As used herein, the term "autologous" is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

"Allogeneic" refers to a graft derived from a different animal of the same species. "Xenogeneic" refers to a graft derived from an animal of a different species.

The term "cancer" as used herein is defined as disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include, but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia (*e.g.*, chronic lymphocytic leukemia, acute lymphoblastic leukemia, pediatric acute B cell leukemia, or post hematopoietic stem cell transplant relapsed leukemia), lung cancer and the like. In some embodiments, cancer refers to B-cell related malignancies (*e.g.*, B-cell chronic lymphocytic leukemia, acute lymphoblastic leukemia, B-cell prolymphocytic leukemia, precursor B lymphoblastic leukemia, Hairy cell leukemia,

diffuse large B cell lymphoma, follicular lymphoma, marginal zone lymphoma, small cell lymphocytic lymphoma, mantle cell lymphoma, Burkitt lymphoma, primary mediastinal (thymic) large B-cell lymphoma, lymphoplasmacytic lymphoma, nodal marginal zone B cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, lymphomatoid
5 granulomatosis, or plasmablastic lymphoma).

In some embodiments, cancer refers to CD30+ cancers (*e.g.*, Hodgkin's lymphoma (HL), anaplastic large cell lymphoma (ALCL), diffuse large B cell lymphoma, and peripheral T/NK cell lymphoma, primary effusion lymphoma, adult T-cell leukemia/lymphoma, mycosis fungoides, and extranodal natural killer/T-cell lymphoma).

10 In some embodiments, the cancer is a GD2-expressing cancer. In some embodiments, the cancer is retinoblastoma or osteosarcoma. Retinoblastoma is a cancer that develops from cells of the retina and is the most common malignant tumor in children. Retinoblastoma can be identified by the skilled practitioner, *e.g.*, using techniques known in the art including red reflex, Hirschberg test, an eye examination, computerized tomography (CT), magnetic
15 resonance imaging (MRI), or ultrasound. Genetic testing, *e.g.*, mutations in the RB1 gene may also be used to identify subjects. Osteosarcoma is a tumor that occurs in the bone which is of mesenchymal origin. Osteosarcoma occurs most often in children and young adults. Osteosarcoma can be identified by the skilled practitioner, *e.g.*, using techniques known in the art including X-ray, CT scan, PET scan, bone scan, MRI, or biopsy.

20 The term "autoimmune disease" as used herein refers to a disease characterized by an abnormal immune response of the body against the body's own cells and tissues. The immune response may be systemic or may be restricted to certain tissue types or organs. Examples of various autoimmune diseases include, but are not limited to, acute disseminated encephalomyelitis, Addison's disease, alopecia areata, autoimmune cardiomyopathy,
25 autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune lymphoproliferative syndrome, autoimmune peripheral neuropathy, autoimmune pancreatitis, autoimmune polyendocrine syndrome, autoimmune progesterone dermatitis, autoimmune thrombocytopenic purpura, autoimmune urticarial, autoimmune uveitis, Behçet's disease, Celiac disease, Churg-Strauss syndrome, cold agglutinin disease, Crohn's disease,

dermatomyositis, diabetes mellitus type 1, eosinophilic fasciitis, gastrointestinal pemphigoid, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome, Hashimoto's encephalopathy, Hashimoto's thyroiditis, idiopathic thrombocytopenic purpura, lupus erythematosus, Miller-Fisher syndrome, mixed connective tissue disease, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, pernicious anaemia, polymyositis, primary biliary cirrhosis, psoriasis, psoriatic arthritis, relapsing polychondritis, rheumatoid arthritis, rheumatic fever, Sjögren's syndrome, temporal arteritis, transverse myelitis, ulcerative colitis, undifferentiated connective tissue disease, vasculitis, and Wegener's granulomatosis.

The term "infection" as used herein refers to an invasion of a host's cells or tissues with an infectious organism, such as a bacteria, virus, or fungus.

An "effective amount" as used herein, means an amount which provides a therapeutic or prophylactic benefit.

As used herein, the term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

"Expression vector" refers to a vector 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, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

The term "immunoglobulin" or "Ig," as used herein is defined as a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of proteins are IgA, IgG, IgM, IgD, and IgE. IgA is the primary antibody that is present in body secretions, such as saliva, tears, breast milk, gastrointestinal secretions and mucus secretions of the respiratory and genitourinary tracts. IgG is the most common circulating antibody. IgM is the main immunoglobulin produced in the primary immune response in most subjects. It is the most efficient immunoglobulin in agglutination, complement fixation, and other

antibody responses, and is important in defense against bacteria and viruses. IgD is the immunoglobulin that has no known antibody function, but may serve as an antigen receptor. IgE is the immunoglobulin that mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to allergen.

5 "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.

10 Unless otherwise specified, a "nucleotide sequence or nucleic acid 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 an RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

15 A "lentivirus" as used herein refers to a genus of the Retroviridae family. Lenti viruses 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 lenti viruses. Vectors derived from lenti viruses offer the means to achieve
20 significant levels of gene transfer *in vivo*.

By the term "modulating," as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing
25 and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

"Codon-optimized" means that codons relating to a specific amino acid are optimized for translational efficiency of a gene of interest. Codon optimization typically involves evaluating the gene or sequence of interest and substituting the codon with a more prevalent

or common codon used for the same amino acid in a specific cell or species. Programs used by those in the art to evaluate codon optimization include those provided by Integrated DNA Technologies, EnCor Biotechnology, Inc., JCat, OptimumGene TM (GenScript USA, Inc., Pisataway, NJ 08854), *etc.* The sequences encoding the CAR embodiments described herein
5 may be codon-optimized, which can increase their translational efficiency.

The term "operably linked" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid
10 sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The term "overexpressed" or "overexpression" is intended to indicate an abnormal
15 level of expression (*e.g.*, of the tumor antigen) in a cell from a disease area (*e.g.*, a solid tumor within a specific tissue or organ of the patient) relative to the level of expression in a normal cell from that tissue or organ. Patients having solid tumors or a hematological malignancy characterized by overexpression of the tumor antigen can be determined by standard assays known in the art.

20 "Parenteral" administration of an immunogenic composition includes, *e.g.*, subcutaneous (s.c), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or in situ, amenable to the
25 methods described herein. In some embodiments, the patient, subject or individual is a human. In some embodiments, the patient, subject or individual is a child (such as 18 years of age or younger).

The term "promoter" as used herein is defined as 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.

As used herein, the term "promoter/regulatory sequence" means 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 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. A "constitutive" promoter is 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.

An "inducible" promoter is 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.

A "tissue- specific" promoter is a nucleotide sequence which, when 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.

By the term "specifically binds" or "specific for", as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (*e.g.*, an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein

structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

The term "subject" is intended to include living organisms in which an immune response can be elicited (*e.g.*, mammals). Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof.

The term "therapeutic" as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

The term "therapeutically effective amount" refers to the amount of the subject compound that will elicit the biological or medical response of a tissue, system, or subject that is being sought by the researcher, veterinarian, medical doctor or other clinician. The term "therapeutically effective amount" includes that amount of a compound that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the signs or symptoms of the disorder or disease being treated. The therapeutically effective amount will vary depending on the compound, the disease and its severity and the age, weight, *etc.*, of the subject to be treated.

To "treat" a disease as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

The term "transfected" or "transformed" or "transduced" as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated 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 "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate

transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

5 **Compositions**

In some embodiments, the present invention provides a chimeric antigen receptor (CAR) comprising (a) an extracellular domain comprising an antigen binding domain, (b) a transmembrane domain and (c) a cytoplasmic domain. It should be appreciated that in some
 10 arrangements are from left to right, N-terminus to C-terminus of the CAR. A CAR molecule as described by the disclosure may comprise or further comprise any other combination of elements as described herein.

In some embodiments, a CAR as described by the disclosure is fully human. In some
 15 embodiments, a CAR has a cytoplasmic domain comprising, a IL-15R α cytoplasmic domain in combination with one or more other cytoplasmic domains described herein, *e.g.*, a CD3 zeta domain. In some embodiments, the arrangement of the elements of a CAR is selected from one of the following exemplary, non-limiting arrangements:

scFv-CD28- IL-15R α -CD3z

scFv-CD28-CD27-IL-15R α -CD3z

20 scFv-CD28-(4-1BB)-CD27-IL-15R α -CD3z

scFv-CD8-CD27-IL-15R α -CD3z

scFv-CD8-(4-1BB)-CD27-IL-15R α -CD3z

In some embodiments, a CAR has a cytoplasmic domain comprising, a CD27
 25 cytoplasmic domain in combination with one or more other cytoplasmic domains described herein, *e.g.*, a 4-1BB intracellular domain and/or a CD3 zeta domain. In some embodiments, the cytoplasmic domain further comprises a safety-enhancing domain, *e.g.*, an apoptosis-inducing iCasp9-FKBP domain. In some embodiments, the arrangement of the elements of a CAR is selected from one of the following exemplary, non-limiting arrangements:

scFv-CD28-(4-1BB)-CD27- CD3z-iCasp9-FKBP

scFv-CD28-CD27-(4-1BB)-CD3z-iCasp9-FKBP

scFv-CD8-(4-1BB)-CD27- CD3z-iCasp9-FKBP

scFv-CD8- CD27-(4-1BB)-CD3z-iCasp9-FKBP

In some embodiments, a CAR described by the disclosure comprises an antigen binding domain specific for GD2. In some embodiments, the arrangement of the elements of the CAR is selected from one of the following exemplary, non-limiting arrangements:

GD2scFv-CD28-(4-1BB)-CD27-CD3z

GD2scFv-CD8-CD28-CD3z

GD2scFv-CD28-(4-1BB)-CD27-CD3z-Casp9-FKBP

GD2scFv-CD8-CD28-CD3z -Casp9-FKBP

Between the extracellular domain (comprising the antigen binding domain) and the transmembrane domain of the CAR, or between the cytoplasmic domain and the transmembrane domain of the CAR, there may be incorporated a spacer or hinge domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular domain and/or the cytoplasmic domain in the polypeptide chain. As used herein, a hinge domain generally means any oligo- or polypeptide that functions to provide flexibility to the CAR, or domains thereof, and/or prevent steric hindrance of the CAR, or domains thereof. In some embodiments, a spacer or hinge domain may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 5 to 20 amino acids. It also should be appreciated that one or more spacer domains may be included in other regions of a CAR, as aspects of the disclosure are not limited in this respect.

It is to be understood that a CAR can include a region (*e.g.*, an antigen binding domain, a transmembrane domain, a cytoplasmic domain, a signaling domain, a safety domain, and/or a linker, or any combination thereof) having a sequence provided herein or a variant thereof or a fragment of either one thereof (*e.g.*, a variant and/or fragment that retains the function required for the CAR activity) can be included in a CAR protein as described herein. In some embodiments, a variant has 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid changes relative to the illustrated sequence. In some embodiments, a variant has a sequence that is at

least 80%, at least 85%, at least 90%, 90%-95%, at least 95% or at least 99% identical to the illustrated sequence. In some embodiments, a fragment is 1-5, 5-10, 10-20, 20-30, 30-40, or 40-50 amino acids shorter than a sequence provided herein. In some embodiments, a fragment is shorter at the N-terminal, C-terminal, or both terminal regions of the sequence provided. In some embodiments, a fragment contains 80%-85%, 85%-90%, 90%-95%, or 95%-99% of the number of amino acids in a sequence provided herein.

In some embodiments, the spacer and/or hinge sequences of the CAR are selected from one or more of the following exemplary sequences:

Spacer Sequences:

GGGGS (SEQ ID NO: 1)
 GGGGSGGGGS (SEQ ID NO: 2)
 GGGGS x3 (SEQ ID NO: 3)
 GS18: GSTSGGGSGGGSGGGGS (SEQ ID NO: 4)
 218S: GSTSGSGKPGSSEGSTKG (SEQ ID NO: 5)
 GS8: GGGGSGGG (SEQ ID NO: 6)

Hinge Sequences:

Native: VEPKSCDKTHTCPPCP (SEQ ID NO: 7)
 C233S: LDPKSSDKTHTCPPCP (SEQ ID NO: 8)
 C233P: VEPKSPDKTHTCPPCP (SEQ ID NO: 9)
 Delta5: LDKTHTCPPCP (SEQ ID NO: 10)

Antigen binding domains

In some embodiments, the CAR of the invention comprises an antigen binding domain. The choice of binding domain 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, such as cancer or an autoimmune disease. Thus examples of cell surface markers that may act as ligands for the antigen binding domain in the CAR of the invention include those associated with cancer cells and other forms of diseased cells, for example,

autoimmune disease cells and pathogen infected cells. In some embodiments, the CAR of the invention is engineered to target a tumor antigen of interest by way of engineering a desired antigen binding domain that specifically binds to an antigen on a tumor cell. In the context of the present invention, "tumor antigen" refers to antigens that are common to specific

5 hyperproliferative disorders such as cancer. The antigens discussed herein are merely included by way of example. The list is not intended to be exclusive and further examples will be readily apparent to those of skill in the art.

The antigen binding domain of the CAR may target, for example, CD19, CD30, or GD2. Other examples of target antigens include, but are not limited, to CD2, CD5, CD7,

10 CD10, CD19, CD20, CD22, CD30, CD33, CD38, CD52, CD56, CD74, CD138, CD317, Her2, VEGFR2, EGFRviii, CXCR4, BCMA, GD2, GD3, and any other antigens over-expressed in target or diseased cells. Other antigens specific for cancer that may be targeted at taught in PCT publication No. WO2013/123061 (page 20), which is incorporated herein by reference with respect to the antigens recited therein.

15 The antigen binding domain can be any domain that binds to the antigen including but not limited to monoclonal antibodies, scFvs, polyclonal antibodies, synthetic antibodies, human antibodies, humanized antibodies, and fragments thereof. In some instances, it is beneficial for the antigen binding domain to be 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

20 antigen binding domain of the CAR to comprise a human antibody or fragment thereof. Thus, in some embodiments, the antigen binding domain comprises a human antibody or a fragment thereof.

An antigen binding domain (*e.g.*, an scFV) that "specifically binds" to a target or an epitope is a term understood in the art, and methods to determine such specific binding are

25 also known in the art. A molecule is said to exhibit "specific binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular target antigen than it does with alternative targets. An antibody "specifically binds" to a target antigen if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antigen binding domain

(*e.g.*, an scFV) that specifically binds to GD2 or an epitope therein is an antibody that binds this target antigen with greater affinity, avidity, more readily, and/or with greater duration than it binds to other antigens or other epitopes in the same antigen. It is also understood by reading this definition that, for example, an antigen binding domain (*e.g.*, an scFV) that specifically binds to a first target antigen may or may not specifically bind to a second target antigen. As such, "specific binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means specific binding. In some embodiments, antigen binding domains (*e.g.*, scFVs) described herein have a suitable binding affinity to GD2. As used herein, "binding affinity" refers to the apparent association constant or K_A . The K_A is the reciprocal of the dissociation constant (K_D). The antibody described herein may have a binding affinity (K_A) of at least 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} M, or higher. An increased binding affinity corresponds to a decreased K_D . Higher affinity binding of an antibody to a first target relative to a second target can be indicated by a higher K_A (or a smaller numerical value K_D) for binding the first target than the K_A (or numerical value K_D) for binding the second target. In such cases, the antibody has specificity for the first target relative to the second target. Differences in binding affinity (*e.g.*, for specificity or other comparisons) can be at least 1.5, 2, 3, 4, 5, 10, 15, 20, 37.5, 50, 70, 80, 91, 100, 500, 1000, 10,000 or 10^5 fold.

Binding affinity can be determined by a variety of methods including equilibrium dialysis, equilibrium binding, gel filtration, ELISA, surface plasmon resonance, or spectroscopy (*e.g.*, using a fluorescence assay). Exemplary conditions for evaluating binding affinity are in, *e.g.*, TRIS-buffer (50 mM TRIS, 150 mM NaCl, 5 mM CaCl₂ at pH7.5). These techniques can be used to measure the concentration of bound binding protein as a function of target protein concentration. The concentration of bound binding protein ([Bound]) is related to the concentration of free target protein ([Free]) and the concentration of binding sites for the binding protein on the target where (N) is the number of binding sites per target molecule by the following equation:

$$[\text{Bound}] = [N][\text{Free}]/(K_d + [\text{Free}])$$

It is not always necessary to make an exact determination of K_A , though, since sometimes it is sufficient to obtain a quantitative measurement of affinity, *e.g.*, determined using a method such as ELISA or FACS analysis, is proportional to K_A , and thus can be used for

5 comparisons, such as determining whether a higher affinity is, *e.g.*, 2-fold higher, to obtain a qualitative measurement of affinity, or to obtain an inference of affinity, *e.g.*, by activity in a functional assay, *e.g.*, an *in vitro* or *in vivo* assay.

For *in vivo* use of antibodies in humans, it may be preferable to use human antibodies. Completely human antibodies are particularly desirable for therapeutic treatment of human

10 subjects. Human antibodies can be made by a variety of methods known in the art including phage display methods using antibody libraries derived from human immunoglobulin sequences, including improvements to these techniques. See, also, U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, W098/16654, WO 96/34096, WO 96/33735, and WO91/10741; each of which is incorporated

15 herein by reference in its entirety. A human antibody can also be an antibody wherein the heavy and light chains are encoded by a nucleotide sequence derived from one or more sources of human DNA. Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain

20 immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the

25 introduction of human immunoglobulin loci by homologous recombination. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to

produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a polypeptide of the invention.

Antibodies directed against an antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies, including, but not limited to, IgG1 (gamma 1) and IgG3. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, *e.g.*, PCT Publication Nos. WO2014/055771, WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; and 5,939,598, each of which is incorporated by reference herein in their entirety.

A "humanized" antibody retains a similar antigenic specificity as the original antibody, *i.e.*, in the present invention, the ability to bind an antigen described herein, for example, CD19, CD30, or GD2.

In some embodiments, the antigen binding domain of the CAR of the invention targets CD19. In some embodiments, the antigen binding moiety portion in the CAR of the invention is a fully human anti-CD19 scFV. In some embodiments, the anti-CD19 scFV comprises the sequence below, or the complementarity determining regions (CDRs, underlined below and numbered CDR1-3) contained within the sequence below.

Exemplary CD19 scFv:

Light chain:

DIQMTQTTSSLSASLGDRVTISC RASQDISKYLN (CDR1) WYQQKPDGTVKLLIY
HTSRLHS (CDR2) GVPSRFSGSGSGTDYSLTISNLEQEDIATYFC QQGNTLPYT
(CDR3) FGGGTKLEIT (SEQ ID NO: 11)

Heavy chain:

EVKLQESGPGLVAPSQSLSVTCTVSGVSLP DYGVS (CDR1) WIRQPPRKGLEWLG
VIWGSETTY (CDR2) YNSALKSRLTIHKDNSKSQVFLKMNSLQTDDTAIYYCAK
HYYYGGSYAMDY (CDR3) WGQGTSVTVSS (SEQ ID NO: 12)

5

In some embodiments, the antigen binding domain of the CAR of the disclosure targets CD30. In some embodiments, the antigen binding moiety portion in the CAR of the disclosure is a fully human anti-CD30 scFv. In some embodiments, the anti-CD30 scFv comprises a sequence below, or the complementarity determining regions (CDRs, underlined below and numbered CDR1-3) contained within the sequences below:

10

Exemplary CD30 scFvs:*AC10 scFv Light chain:*

DIVMTQSPDSLAVSLGERATINC KASQSVDFDGD SYMN (CDR1)
 15 WYQQKPGQPPKLLIY AASNLES (CDR2)
GVPDRFSGSGSGTDFTLTISLQAEDVAVYYCQQSNEDPWT (CDR3) FGQGTKVEIK
 (SEQ ID NO: 13)

AC10 scFv Heavy chain:

20 VHQLVQSGAEVKKPGASVKVCKAS GYTFTDYYIT (CDR1)
 WVRQAPGQGLEWMG WIYPGSGNTKYNEKFKG (CDR2)
RVTMTRDTSISTAYMELSRRLSDDTAVYYCANYGNYWFAY (CDR3)
 WGQGTLLVTVSS (SEQ ID NO: 14)

5F11 scFv Light Chain:

25 DIQMTQSPTSLASVGDRVITTC RASQGISSWLT (CDR1) WYQQKPEKAPKSLIY
AASSLQS (CDR2) GVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYDSYPIT (CDR3)
 FGQGTRLEIK (SEQ ID NO: 15)

5F11 scFv Heavy Chain:

QVQLQQWGAGLLKPSETLSLTCAVYGGSF YYYWS (CDR1) WIRQPPGKGLEWIG
DINHGGGTNYNPSLKS (CDR2)
RVTISVDTSKNQFSLKLNSVTAADTAVYYCASLTAY (CDR3) WGQGSLVTVSS (SEQ
 5 ID NO: 16)

In some embodiments, the antigen binding domain of the CAR of the invention is specific for GD2. In some embodiments, the antigen binding moiety portion in the CAR of the invention is an anti-GD2 scFV, such as a fully human anti-GD2 scFV. In some
 10 embodiments, the anti-GD2 scFV comprises the sequence(s) of the light and/or heavy chain variable regions of hu3F8, c.60C3 or hu14.18, or the complementarity determining regions (CDRs) contained within the light and/or heavy chain variable regions of hu3F8, c.60C3 or hu14.18 (see, *e.g.*, Yu, A.L., et al., Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. N Engl J Med, 2010. 363(14): p. 1324-34; Ahmed, M. and
 15 N.K. Cheung, Engineering anti-GD2 monoclonal antibodies for cancer immunotherapy. FEBS Lett, 2014. 588(2): p. 288-97; and Alvarez-Rueda, N., et al., Binding activities and antitumor properties of a new mouse/human chimeric antibody specific for GD2 ganglioside antigen. Clin Cancer Res, 2007. 13(18 Pt 2): p. 5613s-5620s). In some embodiments, the anti-GD2 scFV comprises the variable heavy chain (VH) and variable light chain (VL)
 20 sequences below, or the complementarity determining regions (CDRs, underlined below) contained within the sequences below.

Exemplary GD2 scFV:

25 *VH: (CDR regions underlined)*

QVQLVESGPGVVQPGRSLRISCAVSGFSVT NYGVH WVRQPPGKGLEWLG
VIWAGGITNYNSAFMS RLTISKDNSKNTVYLQMNSLRAEDTAMYYCAS
RGGHYGYALDY WGQGTSLVTVSS (SEQ ID NO: 17)

VL: (CDR regions underlined)

EIVMTQTPATLSVSAGERVTITC KASQSVSNDVT WYQQKPGQAPRLLIY
SASNRYSGVPARFSGSGYGTEFTFTISSVQSEDFAVYFC QQDYSS
 FGQGTKLEIK (SEQ ID NO: 18)

5

Other exemplary anti-GD2 scFV sequences are shown below with an exemplary 218S linker sequence underlined.

hu3F8 scFv: (VH and VL linked by 218S linker)

10

QVQLVESGPGVVQPGRSLRISCAVSGFSVTNYGVHWVRQPPGKGLEWLGVIWAGGI
 TNYNSAFMSRLTISKDNSKNTVYQLQMNSLRAEDTAMYYCASRGGHYGYALDYWG
 QGTLVTVSSSGTSGSGKPGSSEGSTKGEIIVMTQTPATLSVSAGERVTITCKASQSVSN
 DVTWYQQKPGQAPRLLIYSASNRYSGVPARFSGSGYGTEFTFTISSVQSEDFAVYFCQ
 QDYSSFGQGTKLEIK (SEQ ID NO: 19)

15

C60c3 ScFv: (VH and VL linked by 218S linker)

EVKLVESGGGLVLPGLSLRLSCATSEFTFTDYMTWVRQPPRKALEWLGFIIRNAN
 GYTTEYNPSVKGRFTISRDNQSILYLQMNTLRTEDSATYYCARVSNWAFDYWGQG
 TTLTVSSSGTSGSGKPGSSEGSTKGDVVMTQTPLSLPVSLGDQASISCRSSQSLLKNN
 20 GNTFLHWYLQKSGQSPKLLIYKVSNRSLSGVPDRFSGSGSGTYFTLKISRVEAEDLGV
 YFCSQSTHIPYTFGGGTKLEIK (SEQ ID NO: 20)

Hu14.18 scFv: : (VH and VL linked by 218S linker)

EVQLVQSGAEVEKPGASVKISCKASGSSFTGYNMNWVRQNIGKSLEWIGAIIDPYYG
 25 GTSYNQKFKGRATLTVDKSTSTAYMHLKSLRSEDVAVYYCVSGMEYWGQGTSVTV
 SSGTSGSGKPGSSEGSTKGDVVMTQTPLSLPVTPGEPASISCRSSQSLVHRNGNTYL
 HWYLQKPGQSPKLLIHKVSNRFSGLVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQS
 THVPPLTFGAGTKLELK (SEQ ID NO: 21)

Further Extracellular domain

In some embodiments, the CAR is designed to include an extracellular T cell co-stimulatory domain such as CD28 extracellular domain, or a portion thereof. The extracellular domain may serve as a hinge domain or T cell activation domain. Examples include the CD28 extracellular domain, which has 50 amino acids. An exemplary sequence of the CD28 extracellular domain is:

YVNQTDIYFCKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFLPGPSKP (SEQ ID NO: 22).

Transmembrane domain

With respect to the transmembrane domain, the CAR can be designed to comprise a transmembrane domain that is fused to the extracellular domain (*e.g.*, the antigen binding domain) of the CAR. Any transmembrane domain is contemplated for use herein as long as the domain is capable of anchoring a CAR comprising the domain to a cell membrane. In some embodiments, the transmembrane domain that naturally is associated with one of the domains in the CAR is used. 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 to minimize interactions with other members of the receptor complex. One skilled in the art would appreciate that the full transmembrane domain, or portion thereof, is implemented with the cytoplasmic domain, or a portion thereof. Typically, the transmembrane and cytoplasmic domains used would be contiguous portions of the CD28 sequence.

The transmembrane domain may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane domains of particular use in this invention may be derived from (*e.g.*, comprise at least the transmembrane domain(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, and CD154. Transmembrane domains can be identified using any method known in the art or described herein, *e.g.*, by

using the UniProt Database.

In some embodiments, the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic
 5 transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

In some embodiments, the transmembrane domain in the CAR of the invention is the
 10 CD8 transmembrane domain. Sequences of CD8 for this purposes are taught in PCT pub no. WO2014/055771.

In some embodiments, the transmembrane domain in the CAR of the invention is a CD28 transmembrane domain. An exemplary sequence of CD28 is provided below, as well as an exemplary transmembrane domain sequence. In some embodiments, the CD28
 15 transmembrane domain comprises the exemplary transmembrane domain sequence below, or a fragment or variant thereof that is capable of anchoring a CAR comprising the sequence to a cell membrane.

CD28 (amino acids 19-220)

20 NKILVKQSPMLVAYDNAVNLSCKYSYNLFSREFRASLHKGLDSAVEVCVVYGNYSQQLQV
 YSKTGFNCDGKLGNE SVTFYLQNL YVNQTDIYFCKIEVMYPPPYLDNEKSNGTIIHVKGK
 HLCPSPLFPGPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRR
 PGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 23)

25 CD28 (amino acids 153-179, transmembrane domain)

FWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO: 24)

In some embodiments, the CAR of the invention is comprises a region of CD28 that contains all or part of an extracellular domain, all or part of a transmembrane domain and all
 30 or part of a cytoplasmic domain. An exemplary sequence of a region of CD28 for inclusion

in a CAR is provided below. In some embodiments, the CD28 transmembrane domain comprises the exemplary transmembrane domain sequence below, or a fragment or variant thereof that is capable of anchoring a CAR comprising the sequence to a cell membrane.

5 CD28 region

IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFLPGPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSAS (SEQ ID NO: 25)

10 In some embodiments, the transmembrane domain of the CAR of the invention comprises a hinge domain such as a CD8 hinge domain. An exemplary CD8 hinge domain sequence is provided below. In some embodiments, the CD8 hinge domain comprises the exemplary sequence below, or a fragment or variant thereof that is capable of providing flexibility to or preventing steric hindrance of the CAR or the domain(s) attached to the hinge domain. In some instances, a variety of human hinges can be employed as well including the human Ig (immunoglobulin) hinge.

CD8 hinge domain

AKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO: 26)

Cytoplasmic domain

25 In some embodiments, the cytoplasmic domain or otherwise the intracellular signaling domain of the CAR of the invention is responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been placed in. 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 including the secretion of cytokines. Thus the term "intracellular signaling domain" refers to the portion of a protein which transduces the effector function signal and directs the cell to perform a specialized function.

While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire domain. 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 domain 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 some embodiments, the cytoplasmic domain comprises an IL-15R α cytoplasmic domain. In some embodiments, the intracellular IL-15R α cytoplasmic domain displays effector signaling function that enhances immune effector activities including, but not limited to cell proliferation and cytokine production. An exemplary IL-15R α cytoplasmic domain sequence is provided below. In some embodiments, the IL-15R α cytoplasmic domain comprises the exemplary sequence below, or a fragment or variant thereof that, when included in a CAR, has the same or an improved function (such as cytolytic activity, cell proliferation or secretion of cytokines) compared to a CAR comprising the exemplary sequence below. The function may be tested using a method provided herein, such as the method provided in Example 1.

IL-15R α intracellular domain

KSRQTPPLASVEMEAMEALPVTWGTSSRDEDLNCSHHL (SEQ ID NO: 27)

In some embodiments, the cytoplasmic domain comprises a CD27 intracellular domain (*e.g.*, CD27 cytoplasmic domain). In some embodiments, the intracellular CD27 cytoplasmic domain displays effector signaling function that enhances immune effector activities including, but not limited to cell proliferation and cytokine production. An exemplary CD27 cytoplasmic domain sequence is provided below. In some embodiments, the CD27 cytoplasmic domain comprises the exemplary sequence below, or a fragment or variant thereof that, when included in a CAR, has the same or an improved function (such as cytolytic activity, cell proliferation or secretion of cytokines) compared to a CAR comprising the exemplary sequence below. The function may be tested using any suitable method

known in the art.

CD27 intracellular domain

QRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (SEQ ID NO: 38)

5

Examples of other intracellular signaling domains for use in the CAR of the invention 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 fragment or variant of these sequences and any synthetic sequence that has the same functional capability.

10

It is known that signals generated through the endogenous TCR alone are insufficient for full activation of the T cell and that a secondary or co-stimulatory signal is also required. Thus, T cell activation can be mediated by two distinct classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences) and those that act in an antigen- independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences).

15

Primary cytoplasmic signaling sequences regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing primary cytoplasmic signaling sequences that are of particular use in the invention include those derived from TCR zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. It is particularly preferred that cytoplasmic signaling molecule in the CAR of the invention comprises a cytoplasmic signaling sequence derived from CD3zeta. Exemplary CD3 zeta domain sequences are provided below. In some embodiments, the CD3zeta signaling domain comprises one of the exemplary sequences below, or a fragment or variant thereof that, when included in a CAR, has the same or an improved function (such as cytolytic activity or secretion of cytokines) compared to a CAR comprising the exemplary sequence below. The function may be tested using a method

20

25

provided herein, such as the method provided in Example 1.

CD3 zeta signaling domain

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
 5 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
 PR (SEQ ID NO: 39)

CD3 zeta signaling domain

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
 10 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
 PR (SEQ ID NO: 40)

The cytoplasmic domain of the CAR can be designed to comprise a CD3-zeta signaling domain combined with any other desired cytoplasmic domain(s) useful in the context of the CAR of the invention. For example, the cytoplasmic domain of the CAR can
 15 comprise a CD3 zeta domain and a costimulatory signaling region. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. Thus, while the invention is exemplified primarily with 4-1BB, CD28, and CD27 as the co-stimulatory signaling element, other additional costimulatory
 20 elements are within the scope of the invention. Exemplary co-stimulatory signaling regions include 4-1BB, CD21, CD28, CD27, CD127, ICOS, IL-15R α , and OX40.

In some embodiments, the cytoplasmic domain of a CAR can be designed to comprise an IL-15R α cytoplasmic domain and a CD3-zeta signaling domain combined with any other desired cytoplasmic domain(s) useful in the context of the CAR of the invention. For
 25 example, the cytoplasmic domain of the CAR can comprise an IL-15R α cytoplasmic domain, a CD3 zeta domain and a costimulatory signaling region. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. Thus, while the invention is exemplified primarily with 4-1BB, CD28, CD137 and CD27 as the co-stimulatory signaling element, other additional costimulatory elements are

within the scope of the invention.

The cytoplasmic domain of the CAR can be designed to comprise CD27 cytoplasmic domain and a CD3-zeta signaling domain combined with any other desired cytoplasmic domain(s) useful in the context of the CAR of the disclosure. For example, the cytoplasmic domain of the CAR can comprise CD27 cytoplasmic domain, a CD3 zeta domain and a costimulatory signaling region. The costimulatory signaling region refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. Thus, while the disclosure is exemplified primarily with IL-15R α , 4-1BB, CD28, CD137 and CD27 as the co-stimulatory signaling element, other additional costimulatory elements are within the scope of the disclosure. Example sequences of co-stimulatory signaling regions are shown below.

CD28 (amino acids 180-220, cytoplasmic domain)

RSKRSRLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO: 41)

4-1BB (CD137) intracellular TRAF binding domain

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO: 42)

ICOS intracellular domain

CWLTKKKYSSSVHDPNGEYMFMRVNTAKKSRLTDVTL (SEQ ID NO: 43)

OX40 intracellular domain

ALYLLRRDQRLPPDAHKKPPGGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO: 44)

CD27 intracellular domain

QRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (SEQ ID NO: 38)

CD127 intracellular domain

KRIKPIVWPSLPDHKKKTLEHLCKKPRKNLNVSFNPESFLDCQIHRVDDIQARDEVEGF
LQDTPPQQLEESEKQRLGGDVQSPNCPSADVITPESFGRDSSLTCLAGNVSACDAPI

LSSRSRLDCRESGKNGPHVYQDLLLSLGTNSTLPPFSLQSGILTLNPVAQQQPILTS
GSNQEEAYVTMSSFYQNN (SEQ ID NO: 45)

IL-15R α intracellular domain

5 KSRQTPPLASVEMEAMEALPVTWGTSSRDEDLNCSHL (SEQ ID NO: 27)

In some embodiments, CAR of the invention comprises the apoptosis inducing gene Casp9 or a domain or truncated version thereof. An exemplary Casp9 sequence and truncated sequence is below. In some embodiments, the CAR comprises a 2A peptide linker
10 between a CD3 zeta domain and Casp9.

CASP9 amino acid sequence

MDEADRRLRLRRLVEELQVDQLWDALLSRELFRPHMIEDIQRAGSGSRRDQAR
QLIIDLETRGSQALPLFISCLEDTGQDMLASFLRTNRQAALSKPTLENLTPVVLRLPEI
15 RKPEVLRPETPRPVDIGSGGFGDVGALESRLGNADLAYILSMEPCGHCLIINNVNFCR
ESGLRTRTGSNIDCEKLRRRFSSLFHFMVEVKGDLTAKKMVLALLELAQQDHGALDC
CVVVILSHGCQASHLQFPGAVYGTGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQAC
GGEQKDHGFEVASTSPEDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYST
FPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGC
20 FNFLRKKLFFKTS (SEQ ID NO: 28)

A truncated CASP9 amino acid sequence

VGALESRLGNADLAYILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRRRFSS
LFHFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
25 TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN
PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (SEQ ID NO:
29)

In some embodiments, the CAR further comprises a mutated FK506 binding protein (*e.g.*, FKBPf36v) motif. An exemplary mutated FK506 binding protein motif is provided below.

5 FKBP f36v amino acid sequence

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSSRDRNKPFFKMLGKQE
VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE (SEQ
ID NO: 30)

10 The cytoplasmic signaling sequences within the cytoplasmic signaling portion of the CAR of the invention may be linked to each other in a random or specified order. Optionally, a short oligo- or polypeptide linker or spacer, preferably between 5 and 20 amino acids in length may be inserted between cytoplasmic domains. A GGGGS (SEQ ID NO: 1) or (GGGGS)₃ (SEQ ID NO: 3) provides a particularly suitable linker.

15 In some embodiments, a CAR comprises or consists of the sequence below, which is broken down by exemplary domains included therein (domain names appear in bold after each domain in the exemplary CARs):

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGV
PSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGSTSGSGKPG
20 SGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLG
VIWGSETTYYNALKSRLTIHKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYA
MDYWGQGTSVTVSSAA (**CD19 scFv domain**)

IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFPGPSKPFVVLVVVGGVLACYSLLV
VAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSAS (**CD28**

25 **transmembrane/cytoplasmic domain**)

GGGGSGGGGS (**Linker**)

KSRQTPPLASVEMEAMEALPVTWGTSSRDEDLNCSHHL (**IL-15Ralpha cytoplasmic
signal domain**)

GGGGSGGGGS (**Linker**)

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPPEMGGKPRRKNPQ
 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
 PR (**CD3zeta signal domain**) (SEQ ID NO: 31)

In some embodiments, a CAR comprises or consists of the sequence below, which is
 5 broken down by exemplary domains included therein (domain names appear in bold after
 each domain in the exemplary CARs):

QVQLQQWGAGLLKPSETLSLTCAVYGGSFSAYYWSWIRQPPGKGLEWIGDINHGGG
 TNYNPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCASLTAYWGQGS�TVSS
 (**CD30 scFv domain**)

10 GSTSGSGKPGSSEGSTKG (**Linker**)

DIQMTQSPTSLASVGDRVITICRASQGSSWLTWYQQKPEKAPKSLIYAASSLQSGV
 PSRFSGSGSGTDFTLTISLQPEDFATYYCQQYDSYPITFGQGRLEIK (**CD30 scFv
 domain**)

GSTSGSGKPGSSEGSTKG (**Linker**)

15 FWVLVVVGGVLACYSLLVTVAFIIFWV (**CD28 transmembrane domain**)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL (**4-1BB intracellular
 domain**)

QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (**CD27
 intracellular domain**)

20 GSTSGSGKPGSSEGSTKG (**Linker**)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPPEMGGKPRRKNPQ
 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
 PR (**CD3 zeta domain**)

GSTSGSGKPGSSEGSTKG (**Linker**)

25 VGALES LRGNADLAYILSMEPCGHCLIINN VNF CRESGLRTRTGSNIDCEKLRRRFSSL
 HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
 TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN
 PEPDATPFQEGLRTFDQLDAISSLTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
 FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (**truncated**)

iCasp9 domain)

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFFMLGKQE
 VIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKLE

(FKBP domain) (SEQ ID NO: 32)

5 In some embodiments, the above exemplary, non-limiting arrangements are from left to right, N-terminus to C-terminus of the CAR. The CAR may comprise or further comprise any other combination of elements as described herein.

In some embodiments, a CAR comprises or consists of one the exemplary sequences below, which is broken down by exemplary domains included therein (domain names appear
 10 in bold after each domain in the exemplary CARs):

CAR 1

QVQLVESGPGVVQPGRSLRISCAVSGFSVTNYGVHWVRQPPGKGLEWLGVWAGGI
 TNYNSAFMSRLTISKDNSKNTVYLMNSLRAEDTAMYYCASRGGHYGYALDY
 15 WGQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
 TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC
 DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
 VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
 KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
 20 YKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVVFSCSV MHEALHNHYTQKSLSLSPG
K (GD2 scFv heavy chain)

GSTSGSGKPGSSEGSTKG (218S Linker)

EIVMTQTPATLSVSAGERVTITCKASQSVSNDVTWYQQKPGQAPRLLIYSASNRYSG
 VPARFSGSGYGTEFTFTISSVQSEDAVYFCQQDYSSFGQGTKLEIKRTVAAPS
 25 VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS
 TYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC **(GD2 scFv light chain)**

GSTSGSGKPGSSEGSTKG FWVLVVVGVLACYSLLVTVAFIIFWV (CD28 transmembrane domain)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (**4-1BB intracellular domain**)

QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (**CD27 intracellular domain**)

5 GSTSGSGKPGSSEGSTKG (**218S Linker**)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
EGLYNELQKDKMAEAYSEIGMKGERRRGKGGHDGLYQGLSTATKDTYDALHMQALP
PR (**CD3zeta**)

GSTSGSGKPGSSEGSTKG (**218S Linker**)

10 VGALESLRGNADLAYILSMEPCGHCLINNVNFCRESGLRTRTGSNIDCEKLRRRFSSL
HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN
PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (**truncated**
15 **Casp9 domain**)

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLDGGKKVDSSRDRNKPFFKMLGKQE
VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE
(**FKBP domain**) (SEQ ID NO: 33)

20 CAR 2

QVQLVESGPGVVQPGRSLRISCAVSGFSVTNYGVHWRQPPGKGLEWLGVWAGGI
TNYNSAFMSRLTISKDNSKNTVYLQMNSLRAEDTAMYYCASRGGHYGYALDYWG
QGTLVTVSS (**VH hu3F8**)

GSTSGSGKPGSSEGSTKG (**218S Linker**)

25 EIVMTQTPATLSVSAGERVTITCKASQSVSNDVTWYQQKPGQAPRLLIYSASNRYSG
VPARFSGSGYGTEFTFTISSVQSEDAVYFCQQDYSSFGQGTKLEIK (**VL hu3F8**)
FWVLVVVGGVLACYSLLVTVAFIIFWV (**CD28 transmembrane domain**)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL(**4-1BB intracellular domain**)

QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (CD27
intracellular domain)

GSTSGSGKPGSSEGSTKG(218S Linker)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
5 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
PR (CD3zeta)

GSTSGSGKPGSSEGSTKG (218S Linker)

VGALESLRGNADLAYILSMEPCGHCLIINNPNFCRESGLRTRTGSNIDCEKLRRRFSSL
HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
10 TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN
PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (truncated
Casp9)

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFGFMLGKQE
15 VIRGWEEGVAQMSVGQRAKLTIAPDYAYGATGHPGIIPPHATLVFDVELLKLE
(FKBP domain) (SEQ ID NO: 34)

CAR 3

EVKLVESGGGLVLPGLSLRLSCATSEFTFTDYYMTWVRQPPRKALEWLGFIIRNAN
20 GYTTEYNPSVKGRFTISRDNQSILYLQMNTLRTEDSATYYCARVSNWAFDYWGQG
TTLTVSS (VH C60c3)

GSTSGSGKPGSSEGSTKG (218S Linker)

DVVMQTQPLSLPVSLGDQASISCRSSQSLLKNNGNTFLHWYLQKSGQSPKLLIYKVS
NRLSGVPDRFSGSGSGTYFTLKISRVEAEDLGVYFCSQSTHIPYTFGGGTKLEIK(VL
25 C60c3)

FWVLVVVGGLVACYSLLVTVAFIIFWV (CD28 transmembrane domain)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL(4-1BB intracellular
domain)

QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (CD27

intracellular domain)

GSTSGSGKPGSSEGSTKG(218S Linker)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP

5 PR (CD3zeta)

GSTSGSGKPGSSEGSTKG (218S Linker)

VGALESLRGNADLAYILSMEPCGHCLIINNPNFCRESGLRTRTGSNIDCEKLRRRFSSL
HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN
10 PEPDATPFQEGLRTFDQLDAISSLTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (truncated**Casp9)**MGVQVETISPGDGRTPKRGQTCVVHYTGMLDGGKVDSSRDRNKPFGMLGKQE
VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE

15 (FKBP domain) (SEQ ID NO: 35)

CAR 4EVQLVQSGAEVEKPGASVKISCKASGSSFTGYNMNWVRQNIGKSLEWIGAIIDPYYG
GTSYNQKFKGRATLTVDKSTSTAYMHLKSLRSEDVAVYYCVSGMEYWGQGTSVTV

20 SS (VH Hu14.18)

GSTSGSGKPGSSEGSTKG (218S Linker)

DVVMTQTPLSLPVTPGEPASISCRSSQSLVHRNGNTYLHWYLQKPGQSPKLLIHKVS
NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPPLTFGAGTKLELK(VL
Hu14.18)

25 FWVLVVVGGVLACYSLLVTVAFIIFWV (CD28 transmembrane domain)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL(4-1BB intracellular
domain)QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (CD27
intracellular domain)

GSTSGSGKPGSSEGSTKG(218S Linker)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQ
EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
PR (CD3zeta)

5 GSTSGSGKPGSSEGSTKG (218S Linker)

VGALES LRGNADLAYILSMEPCGHCLINNVNFCRESGLRTRTGSNIDCEKLRRRFSSL
HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN
PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI

10 FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (truncated
Casp9)

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFKFMLGKQE
VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE
(FKBP domain) (SEQ ID NO: 36)

15 In some embodiments, the above exemplary, non-limiting arrangements are from left to right, N-terminus to C-terminus of the CAR. The CAR may comprise or further comprise any other combination of elements as described herein.

Vectors

20 In some embodiments, the present invention encompasses a DNA construct comprising sequences of a CAR, wherein the sequence comprises the nucleic acid sequence of an antigen binding domain operably linked to the nucleic acid sequence of transmembrane domain and a cytoplasmic domain. An exemplary cytoplasmic domain that can be used in a CAR of the invention includes but is not limited to the cytoplasmic domain of IL-15R α and
25 the signaling domain of CD3-zeta. In some embodiments, a CAR comprises the intracellular domain of CD28, 4-1BB, and/or CD27 and the signaling domain of CD3-zeta. In some instances, a CAR can further comprise the apoptosis inducing gene Casp9.

In some embodiments, the arrangement of the elements of the CAR is selected from one of the following exemplary, non-limiting arrangements:

scFv-CD28- IL-15R α -CD3z
 scFv-CD28-CD27-IL-15R α -CD3z
 scFv-CD28-(4-1BB)-CD27-IL-15R α -CD3z
 scFv-CD8-CD27-IL-15R α -CD3z
 5 scFv-CD8-(4-1BB)-CD27-IL-15R α -CD3z
 scFv-CD28-(4-1BB)-CD27- CD3z-iCasp9-FKBP
 scFv-CD28-CD27-(4-1BB)-CD3z-iCasp9-FKBP
 scFv-CD8-(4-1BB)-CD27- CD3z-iCasp9-FKBP
 scFv-CD8- CD27-(4-1BB)-CD3z-iCasp9-FKBP
 10 GD2scFv-CD28-(4-1BB)-CD27-CD3z
 GD2scFv-CD8-CD28-CD3z
 GD2scFv-CD28-(4-1BB)-CD27-CD3z-Casp9-FKBP
 GD2scFv-CD8-CD28-CD3z -Casp9-FKBP

In some embodiments, the above exemplary, non-limiting arrangements are from left
 15 to right, N-terminus to C-terminus of the CAR. The CAR may comprise or further comprise
 any other combination of elements as described herein.

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 gene, by deriving the gene from a vector known to include the same, or by
 20 isolating directly from cells and tissues containing the same, using standard techniques.
 Alternatively, the gene of interest can be produced synthetically, rather than cloned.

The present invention also provides vectors in which a DNA of the present invention
 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
 25 and its propagation in daughter cells. Lenti viral 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. In another embodiment, the desired CAR can be expressed in the
 cells by way of transposons.

In brief summary, 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 into eukaryotes. Typical cloning
5 vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence. The expression constructs of the present invention may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. See, *e.g.*, U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference
10 herein in their entireties. In another embodiment, the invention provides a gene therapy vector.

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
15 expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

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. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York), and in other virology and molecular biology manuals. Viruses, which are useful as
20 vectors 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.*, WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

A number of viral based systems have been developed for gene transfer into
25 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, retrovirus vectors are used. A number of retrovirus vectors are known in the art. In some embodiments, lentivirus vectors are used.

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 recently 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.

One example of a suitable 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. Another example of a suitable promoter is Elongation Factor-1a (EF-1a). 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 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 turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionein promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter. In some embodiments, the promoter is a EF-1a promoter.

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 population of cells sought to be transfected or infected through viral vectors. In other
5 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, and fluorescent genes such as GFP, YFP, RFP and the like. In some embodiments, reporter genes
10 or selectable marker genes are excluded from a CAR polypeptide used in a therapy as described herein.

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
15 whose expression is manifested by some easily detectable property, *e.g.*, enzymatic activity, antibiotic resistance or fluorescence. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the 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
20 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.

25 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.*, mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means. In some embodiments, the host cell is a T cell.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and 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. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

Biological methods for introducing a polynucleotide of interest into a host cell include 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.

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).

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.

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.

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 DNA 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 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.

RNA transfection

5 In some embodiments, the genetically modified T cells of the invention are modified through the introduction of RNA (*e.g.*, an mRNA comprises a sequence encoding a CAR as described herein). In some embodiments, an *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
10 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, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. The desired template for *in vitro* transcription is the CAR of the present invention. For example, in some embodiments, the template for the RNA
15 CAR comprises an extracellular domain comprising an anti-CD19 scFv; a transmembrane domain (such as the transmembrane domain of CD28); and a cytoplasmic domain comprises the signaling domain of CD3-zeta and the cytoplasmic domain of IL-15R α . In some embodiments, the template for the RNA CAR comprises an extracellular domain comprising an anti-CD30 scFv; a transmembrane domain (such as the transmembrane domain of CD28);
20 and a cytoplasmic domain comprises a CD27 intracellular domain, a 4-1BB intracellular domain, and the signaling domain of CD3-zeta. In some embodiments, the template for the RNA CAR comprises an extracellular domain comprising an anti-GD2 scFv; a transmembrane domain (such as the transmembrane domain of CD28); and a cytoplasmic domain comprises a CD27 intracellular domain, a 4-1BB intracellular domain, and the
25 signaling domain of CD3-zeta.

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).

5

Genetically Modified Immune Cells

In some embodiments, the CAR sequence(s) (*e.g.*, nucleic acid sequences encoding a CAR as described herein) are delivered into cells (*e.g.*, T cells or NK cells) using a retroviral or lentiviral vector. In some embodiments, the arrangement of the elements of the CAR encoded by the CAR sequence(s) is selected from one of the following exemplary, non-limiting arrangements:

10

scFv-CD28- IL-15R α -CD3z

scFv-CD28-CD27-IL-15R α -CD3z

scFv-CD28-(4-1BB)-CD27-IL-15R α -CD3z

15

scFv-CD8-CD27-IL-15R α -CD3z

scFv-CD8-(4-1BB)-CD27-IL-15R α -CD3z

scFv-CD28-(4-1BB)-CD27- CD3z-iCasp9-FKBP

scFv-CD28-CD27-(4-1BB)-CD3z-iCasp9-FKBP

scFv-CD8-(4-1BB)-CD27- CD3z-iCasp9-FKBP

20

scFv-CD8- CD27-(4-1BB)-CD3z-iCasp9-FKBP

GD2scFv-CD28-(4-1BB)-CD27-CD3z

GD2scFv-CD8-CD28-CD3z

GD2scFv-CD28-(4-1BB)-CD27-CD3z-Casp9-FKBP

GD2scFv-CD8-CD28-CD3z -Casp9-FKBP

25

In some embodiments, the above exemplary, non-limiting arrangements are from left to right, N-terminus to C-terminus of the CAR. The CAR may comprise or further comprise any other combination of elements as described herein.

CAR-expressing retroviral and lentiviral vectors can be delivered into different types of eukaryotic cells as well as into tissues and whole organisms using transduced cells as

carriers or cell-free local or systemic delivery of encapsulated, bound or naked vectors. The method used can be for any purpose where stable expression is required or sufficient.

In another embodiment, the desired CAR can be expressed in the cells (*e.g.*, T cells or NK cells) by way of transposons.

5 The disclosed methods can be applied to the modulation of immune cell (*e.g.*, T cell or NK cell) activity in basic research and therapy, in the fields of cancer, stem cells, acute and chronic infections, and autoimmune diseases, including the assessment of the ability of the genetically modified T cell or NK cell to kill a target cell, *e.g.*, a target cancer cell.

10 The methods also provide the ability to control the level of expression over a wide range by changing, for example, the promoter or the amount of input vector, making it possible to individually regulate the expression level. For example, varying of different intracellular effector/costimulator domains on multiple chimeric receptors in the same cell allows determination of the structure of the receptor combinations which assess the highest level of cytotoxicity against multi-antigenic targets, and at the same time lowest cytotoxicity
15 toward normal cells.

Sources of Immune Cells

 Prior to expansion and genetic modification of the immune cells (*e.g.*, T cells) of the invention, a source of immune cells (*e.g.*, T cells) is obtained from a subject. Immune cells
20 (*e.g.*, T cells) can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. The immune cells (*e.g.*, T cells) may also be generated from induced pluripotent stem cells or hematopoietic stem cells or progenitor cells. In some embodiments of the present invention, any number of
25 immune cell lines, including but not limited to T cell and NK cell lines, available in the art, may be used. In some embodiments of the present invention, immune 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 some embodiments, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product

typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, NK cells, other nucleated white blood cells, red blood cells, and platelets. In some embodiments, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some
5 embodiments of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Again, surprisingly, initial activation steps in the absence of calcium 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,
10 such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics 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^{2+} -free, Mg^{2+} -free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the
15 apheresis sample may be removed and the cells directly resuspended in culture media.

In another embodiment, immune cells (*e.g.*, T cells) are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient or by counterflow centrifugal elutriation. A specific subpopulation of T cells, such as CD3^+ , CD28^+ , CD4^+ , CD8^+ , CD45RA^+ , and
20 CD45RO^+ T cells, can be further isolated by positive or negative selection techniques. For example, in some embodiments, T cells are isolated by incubation with anti-CD3/anti-CD28 (*i.e.*, 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 some embodiments, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes
25 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 preferred embodiment, the time period is 10 to 24 hours. In one preferred embodiment, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. 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 in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immune-compromised individuals. Further, use of longer incubation times can increase the 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. The skilled artisan would recognize that multiple rounds of selection can also be used in the context of this invention. In certain embodiments, it may be desirable to perform the selection procedure and use the "unselected" cells in the activation and expansion process. "Unselected" cells can also be subjected to further rounds of selection.

Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4⁺ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In certain embodiments, it may be desirable to enrich for or positively select for regulatory T cells which typically express CD4⁺, CD25⁺, CD62L^{hi}, GITR⁺, and FoxP3⁺.

Alternatively, in certain embodiments, T regulatory cells are depleted by anti-CTLA4 conjugated beads or other similar method of selection.

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 embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (*i.e.*, increase the concentration of cells), to ensure maximum contact

of cells and beads. For example, in some embodiments, a concentration of 2 billion cells/ml is used. In some embodiments, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. 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 embodiment, 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 some embodiments, the concentration of cells used is 5×10^6 /ml. In other embodiments, the concentration used can be from about 1×10^5 /ml to 1×10^6 /ml, and any integer value in between.

In other embodiments, 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.

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
5 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. In certain embodiments, 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.

10 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 T cell therapy for any number of diseases or conditions that would benefit
15 from T cell therapy, such as those described herein. In some embodiments a blood sample or an apheresis is taken from a generally healthy subject. In certain embodiments, 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 embodiments, the T cells may be expanded, frozen, and used
20 at a later time. In certain embodiments, samples are collected from a patient shortly after diagnosis of a particular disease as described herein but prior to any treatments. In a further embodiment, 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,
25 immunosuppressive agents, such as cyclosporin, 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. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is

important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1991 ; Henderson et al., Immun. 73:316-321, 1991 ; Bierer et al., Curr. Opin. Immun. 5:763-773, 1993). In a further embodiment, the cells are isolated for a patient and frozen for later use in conjunction with (*e.g.*, before, simultaneously or following) bone marrow or stem cell
5 transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cells are isolated prior to and can be frozen for later use for treatment following B-cell ablative therapy such as agents that react with CD20, *e.g.*, Rituxan.

10 In a further embodiment of the present invention, T cells are obtained from a patient directly following treatment. 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
15 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 embodiments, mobilization (for example,
20 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.

25 Activation and Expansion of T Cells

Whether prior to or after genetic modification of the T cells to express a desirable CAR, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694 and 6,534,055.

Generally, the T cells of the invention are 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 co-stimulatory 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. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, 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): 1319-1328, 1999; Garland et al., J. Immunol Meth. 227(1-2):53-63, 1999).

In certain embodiments, the primary stimulatory signal and the co-stimulatory 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 (*i.e.*, in "cis" formation) or to separate surfaces (*i.e.*, in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In some embodiments, the agent providing the co-stimulatory 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 embodiments, both agents can be in solution. In another embodiment, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing 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.

In some embodiments, the two agents are immobilized on beads, either on the same bead, *i.e.*, "cis," or to separate beads, *i.e.*, "trans." 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 co-stimulatory signal is an anti-CD28 antibody or antigen-binding fragment thereof; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In some embodiments, 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 embodiment 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 some embodiments, the ratio of CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In one aspect of the present invention, 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 embodiments of the invention, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than 2:1. In one particular embodiment, a 1:100 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In a further embodiment, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one preferred embodiment, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In another embodiment, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet another embodiment, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

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 embodiments the ratio of cells to particles ranges from 1:100 to 100:1 and any integer values in-between and in further embodiments 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 preferred 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: 1, 2: 1, 3: 1, 4: 1, 5: 1, 6: 1, 7: 1, 8: 1, 9: 1, 10: 1, and 15: 1 with one preferred ratio being at least 1: 1 particles per T cell. In some embodiments, a ratio of particles to cells of 1: 1 or less is used. In one particular embodiment, a preferred particle: cell ratio is 1:5. In further embodiments, the ratio of particles to cells can be varied
5 depending on the day of stimulation. For example, in some embodiments, 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 embodiment, the ratio of particles to cells is 1: 1 on the first day of stimulation and adjusted to 1:5 on the third and
10 fifth days of stimulation. In another embodiment, 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 another embodiment, 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 another embodiment, particles are added on a daily or every other day basis to a final ratio of 1 : 1 on
15 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 further embodiments of the present invention, the cells, such as T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and
20 then the cells are cultured. In an alternative embodiment, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In a further embodiment, the beads and cells are first concentrated by application of a force, such as a magnetic force, resulting in increased ligation of cell surface markers, thereby inducing cell stimulation.

By way of example, cell surface proteins may be ligated by allowing paramagnetic
25 beads to which anti-CD3 and anti-CD28 are attached (3x28 beads) to contact the T cells. In some embodiments the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1: 1) are combined in a buffer, preferably PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used.

For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (i.e., 100%) may comprise the target cell of interest.

Accordingly, any cell number is within the context of the present invention. In certain embodiments, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in some embodiments, a concentration of about 2 billion cells/ml is used. In another embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. 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. Such populations of cells may have therapeutic value and would be desirable to obtain in certain embodiments. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In some embodiments of the present invention, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may be cultured for 21 days. In some embodiments of the invention the beads and the T cells are cultured together for about eight days. In another embodiment, the beads and T cells are cultured together for 2-3 days. 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).

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 ($\frac{3}{4}$, CD4⁺) that is greater than the cytotoxic or suppressor T cell population (T_c, 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 $\frac{3}{4}$ cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of T_c cells. Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of T_H cells may be advantageous. Similarly, if an antigen- specific subset of T_c cells has been isolated it may be beneficial to expand this subset to a greater degree.

Further, in addition to CD4 and CD 8 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 purposes.

Therapeutic Application

In some embodiments, the present invention provides a cell (*e.g.*, T cell) modified to express a CAR comprises an antigen binding domain, a transmembrane domain (such as CD28 transmembrane domain), and a cytoplasmic domain comprising an IL-15R α cytoplasmic domain, optionally combined with CD3-zeta and/or any other cytoplasmic domains described herein. In some embodiments, a cell is modified to express a CAR

comprising an antigen binding domain, a transmembrane domain (such as CD28 transmembrane domain), and a cytoplasmic domain having a CD27 intracellular domain and a 4-1BB intracellular domain, optionally combined with CD3-zeta and/or any other cytoplasmic domains (*e.g.*, an iCasp9-FKBP domain) described herein. In some
5 embodiments, a cell is modified to express a CAR comprising an antigen binding domain (*e.g.*, a scFV specific for GD2), a transmembrane domain (such as CD28 transmembrane domain), and a cytoplasmic domain. Therefore, in some instances, the transduced T cell can elicit a CAR-mediated T-cell response.

In some embodiments, the invention provides the use of a CAR to redirect the
10 specificity of a primary T cell to an antigen, such as a tumor antigen. Thus, in some embodiments, the present invention also provides a method for stimulating a T cell-mediated immune response to a target cell population or tissue in a mammal comprising the step of administering to the mammal a T cell that expresses a CAR, wherein the CAR comprises an antigen binding domain (*e.g.*, CD19 scFV), a transmembrane domain (such as CD28
15 transmembrane domain), and a cytoplasmic domain comprising an IL-15R α cytoplasmic domain, optionally combined with CD3-zeta and/or any other cytoplasmic domains described herein.

In some embodiments, a method for stimulating a T cell-mediated immune response to a target cell population or tissue in a mammal comprising the step of administering to the
20 mammal a T cell that expresses a CAR, wherein the CAR comprises an antigen binding domain (*e.g.*, CD30 scFV), a transmembrane domain (such as CD28 transmembrane domain), and a cytoplasmic domain having a CD27 intracellular domain and a 4-1BB intracellular domain, optionally combined with CD3-zeta and/or any other cytoplasmic domains (*e.g.*, iCasp9 domain and FKBP domain) described herein.

In some embodiments, the disclosure provides a method for stimulating a T cell-
25 mediated immune response to a target cell population or tissue in a mammal comprising the step of administering to the mammal a T cell that expresses a CAR, wherein the CAR comprises an antigen binding domain (*e.g.*, GD2 scFV), a transmembrane domain (such as CD28 transmembrane domain), and a cytoplasmic domain (*e.g.*, comprising a CD28, 4-1BB,

and/or CD27 intracellular domain combined with CD3-zeta and/or any other cytoplasmic domains described herein.

In some embodiments, the present invention includes a type of cellular therapy where T cells are genetically modified to express a CAR and the CAR T cell is infused to a recipient in need thereof. The infused cell is able to kill cells expressing the antigen, *e.g.*, tumor cells, in the recipient. Unlike antibody therapies, CAR T cells are able to replicate *in vivo* resulting in long-term persistence that can lead to sustained tumor control.

In some embodiments, the CAR T cells of the invention can undergo robust *in vivo* T cell expansion and can persist for an extended amount of time.

While the data disclosed herein specifically disclose lentiviral vector comprising (1) anti-CD19 scFv, a CD28 transmembrane domain, a IL-15R α cytoplasmic domain and a CD3-zeta signaling domain, (2) anti-CD30 scFv, a CD28 transmembrane domain, a cytoplasmic domain having a CD27 intracellular domain and a 4-1BB intracellular domain, and a CD3-zeta signaling domain, and (3) anti-GD2 scFv, a CD28 transmembrane domain, a cytoplasmic domain having a CD27 intracellular domain and a 4-1BB intracellular domain, and a CD3-zeta signaling domain, the invention should be construed to include any number of variations for each of the components of the construct as described elsewhere herein. That is, the invention includes the use of any antigen binding domain in the CAR to generate a CAR-mediated T-cell response specific to the antigen binding domain. For example, the antigen binding domain in the CAR of the invention can target a tumor antigen for the purposes of treat cancer.

In some embodiments, the antigen bind domain portion of the CAR of the invention is designed to treat a particular cancer. For example, the CAR may be designed to target CD19 for treating B cell malignancies, or CD30 for treating Hodgkin's lymphoma or certain T cell lymphoma, or GD2 for treating small cell neuroendocrine cancer or small cell lung cancer, and neuronal cancer.

The CAR-modified T cells of the invention may also serve as a type of vaccine for *ex vivo* immunization and/or *in vivo* therapy in a mammal. Preferably, the mammal is a human.

With respect to *ex vivo* immunization, at least one of the following occurs *in vitro*

prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a CAR to the cells, and/or iii) cryopreservation of the cells.

Ex vivo procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (preferably a human) and genetically modified (e.g., transduced or transfected *in vitro*) with a vector expressing a CAR disclosed herein. The CAR-modified cell can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the CAR-modified cell can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

The procedure for *ex vivo* expansion of hematopoietic stem and progenitor cells is described in U.S. Pat. No. 5,199,942, incorporated herein by reference, can be applied to the cells of the present invention. Other suitable methods are known in the art, therefore the present invention is not limited to any particular method of *ex vivo* expansion of the cells. Briefly, *ex vivo* culture and expansion of T cells comprises: (1) collecting CD34+ hematopoietic stem and progenitor cells from a mammal from peripheral blood harvest or bone marrow explants; and (2) expanding such cells *ex vivo*. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used for culturing and expansion of the cells. In addition to using a cell-based vaccine in terms of *ex vivo* immunization, the present invention also provides compositions and methods for *in vivo* immunization to elicit an immune response directed against an antigen in a patient.

Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised, such as individuals having cancer.

The CAR-modified immune cells (e.g., CAR T cells) of the present invention may be administered either alone, or as a composition (e.g., a pharmaceutical composition) in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations. Briefly, pharmaceutical compositions of the present invention may comprise a target cell population 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 of the present invention are preferably formulated for intravenous administration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

When "therapeutic amount" is indicated, the precise amount of the compositions of the present invention to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). It can generally be stated that a pharmaceutical composition comprising the CAR-modified immune cells (*e.g.*, CAR T cells) described herein may be administered at a dosage of 10^4 to 10^9 cells/kg body weight, preferably 10^5 to 10^6 cells/kg body weight, including all integer values within those ranges. T cell compositions may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are commonly known in immunotherapy (see, *e.g.*, Rosenberg et al., New Eng. J. of Med. 319: 1676, 1988). The optimal dosage and treatment regime for a particular patient can readily be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly.

In certain embodiments, it may be desired to administer activated immune (*e.g.*, T cells) to a subject and then subsequently redraw blood (or have an apheresis performed), activate T cells therefrom according to the present invention, and reinfuse the patient with these activated and expanded T cells. This process can be carried out multiple times every

few weeks. In certain embodiments, T cells can be activated from blood draws of from 10cc to 400cc. In certain embodiments, T cells are activated from blood draws of 20cc, 30cc, 40cc, 50cc, 60cc, 70cc, 80cc, 90cc, or 100cc. Not to be bound by theory, using this multiple blood draw/multiple reinfusion protocol may serve to select out certain populations of T cells.

5 The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In some embodiments, the T cell
10 compositions of the present invention are administered to a patient by intradermal or subcutaneous injection. In another embodiment, the T cell compositions of the present invention are preferably administered by i.v. injection. The compositions of T cells may be injected directly into a tumor, lymph node, or site of infection.

 In certain embodiments of the present invention, cells activated and expanded using
15 the methods described herein, or other methods known in the art where T cells are expanded to therapeutic levels, are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or natalizumab treatment for MS patients or efalizumab
20 treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM
25 PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) bone

marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.*,
5 Rituxan. For example, in some embodiments, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

10 In certain embodiments of the present invention, cells activated and expanded using the methods described herein, or other methods known in the art where T cells are expanded to therapeutic levels, or any other compositions described herein, are administered to a patient in conjunction with (*e.g.*, before, simultaneously or following) any number of relevant treatment modalities, including checkpoint inhibitors, such as PD-L1 inhibitors or PD1
15 inhibitors. In some embodiments, the PD-L1 inhibitors or PD1 inhibitors are PD-L1-specific antibodies or PD1-specific antibodies. Exemplary checkpoint inhibitors include, *e.g.*, pembrolizumab (Merck), ipilimumab (Bristol-Myers Squibb), nivolumab (Bristol-Myers Squibb), MPDL3280A (Roche), MEDI4736 (AstraZeneca), MEDI0680 (AstraZeneca), BMS-936559/MDX-1105 (Bristol-Myers Squibb) and MSB0010718C (Merck). Other PD-
20 L1 and PD1 inhibitors are known in the art (see, *e.g.*, Dolan et al. PD-1 pathway inhibitors: changing the landscape of cancer immunotherapy. Cancer Control. 2014 Jul;21(3):231-7). In some embodiments, compositions described herein are administered in conjunction with (*e.g.*, before, simultaneously or following) chemotherapy and/or radiotherapy.

25 The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices. The dose for CAMPATH, for example, will generally be in the range 1 to about 100 mg for an adult patient, usually administered daily for a period between 1 and 30 days. The preferred daily dose is 1 to 10 mg per day although in some instances larger doses of up to 40 mg per

day may be used (described in U.S. Patent No. 6,120,766). Strategies for CAR T cell dosing and scheduling have been discussed (Ertl et al, 2011, Cancer Res, 71:3175-81; Junghans, 2010, Journal of Translational Medicine, 8:55).

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present disclosure to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLES

Example 1: Interleukin 15R α as co-stimulatory domain in CD19 CART cells enhances the anti-leukemia efficacy

Chimeric Antigen Receptor T cells (CARTs) are engineered T cells displaying specificity against tumor antigens, usually based on one or more single chain Fv (scFv) antibody moieties. The initial CAR design consisted of a receptor complex that combined an antigen binding single chain antibody Fv domain (scFv) and a signal transduction domain of T cells (usually CD3 ζ) {Eshhar Z, 1993, Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and gamma or zeta subunits of the immunoglobulins or T cell receptors}. These so called first generation CARs have only limited anti-tumor activities and *in vivo* survival {Kowolik CM1, 2006, CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances *in vivo* persistence and antitumor efficacy of adoptively transferred T cells.;Hwu P1, 1995, *In vivo* antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes.}. In order to enhance killing efficacy and persistence of CARTs, several second and third generation CARs with the addition of one or two co-stimulatory signals such as CD28 and 4-1BB have been generated {Kowolik CM1, 2006, CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances *in vivo* persistence and antitumor efficacy of adoptively transferred T cells.; Savoldo B, 2011, CD28 costimulation

improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients.; Song DG, 2011, *In vivo* persistence`, tumor localization`, and antitumor activity of CAR-engineered T cells is enhanced by costimulatory signaling through CD137 (4-1BB).; Milone MC, 2009, Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy *in vivo*.}

CD19 has proved to be an effective tumor antigen to target B cell malignancies using CARTs. The first success with CD19 targeting CARTs was reported in chronic lymphocytic leukemia patients and adults with refractory B cell leukemias{Porter DL, 2011, Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia.; Brentjens RJ, 2013, CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia.}. The field is moving extremely fast and recently found to be useful in pediatric acute B cell leukemia{Grupp SA, 2013, Chimeric Antigen Receptor-Modified T cells for Acute Lymphoid Leukemia.}. Preliminary results of CD19 targeted CART clinical trials indicate that this therapy is effective in various settings including post hematopoietic stem cell transplant relapsed leukemia using donor T cells engineered with CD19-CAR{Cruz CR, 2013, Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study.; Brentjens RJ, 2011, Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias.; Kochenderfer JN, 2013, Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation.}.

Results of the CART clinical trials conducted so far indicate that several factors may be influencing the performance of CARTs including those related to disease, patient-related factors and last but not least, CART characteristics. The current focus is on generating CARTs with increased efficacy, longer survival and a good safety profile so that they can be applied to a wider range of malignancies including solid tumors which harbor a hostile tumor microenvironment{Maus MV, 2014, Antibody-modified T cells: CARs take the front seat for hematologic malignancies.; CJ., 2014, Chimeric antigen receptor modified T cell therapy for B cell malignancies.}. One of the ways this can be accomplished is by potentiating CAR

signaling and activation of the genetically modified T cells.

Interleukin-15 (IL-15) is a T cell growth factor which shares many similarities with the more ubiquitously known interleukin-2 (IL-2) {Steel, 2012, Interleukin-15 biology and its therapeutic implications in cancer}. It has a trimeric receptor consisting of an IL-15R α chain which is unique to IL-15 and the IL-15 β (commonly designated as IL-2/15 β) along with common gamma chain (γ C) which it shares with IL-2. Both these interleukins are involved in T cell growth, expansion, activation and survival, but there are specific differences between the two. Most importantly, IL-15 does not cause activation induced cell death (AICD) unlike IL-2 {Munger, 1995, Studies evaluating the antitumor activity and toxicity of interleukin-15, a new T cell growth factor: comparison with interleukin-2; Marks-Konczalik, 2000, IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice}. IL-15 is required for differentiation of NK cells, CD8 cells and maintain memory phenotype CD8 cells which is the main defense mechanism against cancer cells {Jakobisiak, 2011, Interleukin 15 as a promising candidate for tumor immunotherapy}. Its receptor IL-15R α is involved in high affinity binding of IL-15 which presents it to IL-2/IL-15 $\beta\gamma$ which in turn leads to the signaling mediated through various pathways including JAK1/3 and STAT3/5 pathways {Budagian, 2006, IL-15/IL-15 receptor biology: a guided tour through an expanding universe; Okada, 2015, STAT3 signaling contributes to the high effector activities of interleukin-15-derived dendritic cells}.

To increase the efficacy of CARTs, extra co-stimulatory and cytokine signaling domains were incorporated to obtain advanced generation of CARs, abrogating the need to administer additional cytokines along with the CART infusion. Based on this approach, a fourth generation CAR was developed with CD27 co-stimulatory domain in addition to CD28 and 4-1BB signaling domains and its therapeutic efficacy was tested in leukemia and lymphoma patients. The study herein reports a novel CAR design incorporating the IL-15R α -signaling domain and the comparison of the new CAR with both the 3rd (19z, with 4-1BB and CD28 domains) and the 4th generation CARs (273z, with 4-1BB, CD27 and CD28 domains). The study herein demonstrates that with the addition of IL-15R α signaling, the CARTs displayed rapid expansion and target killing activities, which could translate into high

therapeutic efficacy against B cell malignancies.

MATERIALS AND METHODS

Cell lines and culture media. RS4;11 and MV4-11 were CD19 positive acute lymphoblastic leukemia and CD19 negative acute mono-myelocytic leukemia cell lines, respectively, which were obtained from American Type Culture Collection (ATCC). Jurkat T cells were purchased from ATCC. All cell lines were maintained in RPMI1640 medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Inc. Norcross, GA) and supplemented with penicillin (100 units) and streptomycin (100 µg). Target cancer cell lines were transduced with lentiviral vectors expressing a green fluorescent protein (wasabi GFP) and the reporter gene positive cells were sorted by flow cytometry. Jurkat T cells were transduced with lentiviral CAR vectors and the copy number of CAR per cell was determined by quantitative PCR using genomic DNA.

Blood donors and primary T cell culture. Buffy coats from anonymous healthy donors (HDs) were purchased from LifeSouth Civitan Blood Center (Gainesville, FL, USA). PBMCs were isolated from buffy coats by gradient density centrifugation in Ficoll- Hypaque (GE Healthcare Bio-Sciences AB, Piscataway, NJ, USA) as previously described{Chang, 1995, Infection and replication of Tat- human immunodeficiency viruses: genetic analyses of LTR and tat mutations in primary and long-term human lymphoid cells}. Blood and bone marrow samples of children with newly diagnosed acute lymphoblastic leukemia were obtained with the assistance of a Hematological Malignancies Bank. T cells were activated using anti-CD3 and anti-CD28 antibody-conjugated magnetic beads or phytohemagglutinin (PHA). The T cells were maintained in TexMACS medium (Miltenyi Biotec Inc, San Diego, CA) supplemented with interleukin-2, -7 and -15 as previously described {Okada, 2015 #33}. Phenotype of the activated cells was verified to confirm T cell purity. After expansion for two to six days, the T cells were transduced with lentiviral CAR vectors.

Lentivector construction and CAR gene transduction.

Lentivectors were generated using the NHP/TYF lentivector system as previously described{Chang, 2005 #181;Wang, 2006 #19}. CAR DNA was chemically synthesized and

cloned into pTYF transducing vector behind human EF1 α promoter. The final lenti-CAR vectors were verified by restriction enzyme mapping and DNA sequencing.

qPCR determination of CAR copies in CARTs. The CAR transgene copy numbers in

CARTs were determined by quantitative SYBR green real time PCR (qRT-PCR) as previously described {Okada, 2015 #33}. Genomic DNA was harvested from CARTs using Promega Wizard genomic DNA purification kit (Promega Corp. Madison, WI). Conditions for the qRT-PCR reaction was conducted as suggested by SABioscience using the MX3000P qPCR system (Stratagene, Agilent Technologies, Santa Clara, CA).

Calcein AM labeling of target cells.

Fresh or thawed leukemia cells obtained from patients were first washed with RPMI. Then 1×10^6 cells were suspended in 196 μ l of RPMI1640 in a non-attachment tube and 4 μ l of 20 μ M Calcein AM (Life Technologies Corp) was added to get a final concentration of 0.4 μ M. The cells were then incubated at 37°C for 1 hour and subsequently washed twice with RPMI1640 with FBS before using for co-culture experiments.

CART killing assay.

Target cells and CARTs were counted using trypan blue staining and a hemocytometer. The cells were co-cultured in 96U plate in effector:target ratios of 1-2:1, or as indicated in each experiment. CART numbers ranged from 5×10^4 to 2×10^5 . The coculture was incubated in TexMACS medium without cytokines. Cells were incubated for 1-2 hours and overnight for short term assays. CART-targeted killing was recorded by quantitative analysis of the shifted side scattered (SSC) population, and early and late apoptotic (annexin V-stained) and Propidium Iodide (PI)-stained (dead) cells {Zhang, 1997, Early detection of apoptosis using a fluorescent conjugate of annexin V}. Samples were run in the LSRII flow cytometer using the DiVa software (BD Biosciences) and results were analyzed using the FlowJo software.

Annexin V and PI staining for Apoptosis.

After 1 to 2 hours of incubation, cells were transferred to 96-V plate and centrifuged at 400g for 5 minutes to remove the media. Then the cell pellet was washed with PBS once. Cells were re-suspended in 30 μ l of staining mix consisting of 0.25 μ l PI (Sigma), 0.5 μ l

Annexin V (BD Bioscience) and 29.25µl of binding buffer (BD Bioscience) and then incubated in the dark for 15 min at room temperature. Subsequently, cells are suspended in 200µl FACS buffer and analyzed in the LSRII flow cytometer using DiVa software (BD Biosciences). Results were evaluated using FlowJo software.

5 **CFSE cell proliferation assay.**

1X10⁵ CARTs were first washed with PBS and then re-suspended in 100µl of PBS.

A working solution of CFSE was prepared in PBS to get a final concentration of 10µM.

Then 100µl of this was added to the cell suspension to get a final CFSE concentration of 5µM. The cells were incubated at 37C in the dark for 15 minutes. Then the cells were

10 washed twice with cold RPMI+10%FBS and again once with Texsmacs medium. These cells were co-cultured with RS4;11 in a effector to target ratio of 1:5 and incubated in the dark at 37C. For positive control, CFSE labeled T cells were stimulated with anti-CD3 and anti-CD28 Abs with cytokine medium, and for negative control, CFSE labeled T cells were treated with mitomycin C. The cells were analyzed by flow for proliferation indicated by

15 CFSE dilution on Day 2 or 3 after surface staining for CD3 to gate the T cells only.

Monoclonal antibodies (mAbs).

Fluorochrome-conjugated mAbs against human IFN-γ (B27, APC), IL-2 (MQ1-17H12, PE) CD8 (SK1, APC-Cy7), CD4 (RPA-T4, PB), CD22 (S-HCL-1, APC), CD27 (M-T271, APC), CD28 (L-293, PerCp-Cy5.5), CD34 (8G12, PE-Cy7), PD1 (EH12.1, PE-Cy7),

20 and CD107a (H4A3, FITC) were purchased from BD Biosciences (San Diego, CA). Anti-CD19 mAb (SJ25C1, PE) was purchased from Caltag laboratories (Life technologies Inc) and anti-CD127 mAb (RDR5, APC-eflour) was purchased from eBiosciences.

Surface staining and Intracellular cytokine staining.

For effector functional analysis, the CARTs were mixed with target in E/T ratio of 1:3

25 overnight with the addition of 1.5µl of FITC-conjugated anti-CD107a Ab. Positive control used T cells (without CAR) stimulated with PMA (1µg/µl) and Ionomycin (1ng/µl) for 1 hour. The intracellular cytokines were immobilized using monensin (6µg/µl) for 6 hours. The samples were then washed, blocked with 10% human and mouse sera for 30 min, stained with anti-CD4 and anti-CD8 Abs for 30 min, fixed and permeabilized with BD Fix/Perm

Buffer, stained with anti- IFN- γ and anti-IL-2 Abs for 1 hour, and then analyzed by flow cytometry. Data was collected on the BD LSRII flow cytometer and analyzed with Flowjo.

Effector cytokine analysis using cytokine bead array.

The BD CBA™ Human Soluble Protein Flex Set System was used to detect concentrations of cytokines IL-2, IL-6, TNF α and IFN γ in the supernatants collected from the CART killing assays on day 1 or 2 of incubation. The CBA system captures a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead was coated with a capture antibody specific for a soluble protein. The detection reagent was a mixture of PE-conjugated Abs, which provided a fluorescent signal in proportion to the amount of bound analyte. First 10 tubes of 50 μ l set standard dilutions were prepared. Then 50 μ l of each unknown sample was added to an assay tube. 50 μ l of the mixed capture beads was added to each assay tube and incubated at room temperature for 1 hr. Then 50 μ l of the PE detection reagent was added and incubated for 2 hr at room temperature. 1 ml of wash buffer was added to each tube and centrifuged at 200g for 5 minutes. After the supernatant was removed, 300 μ l of wash buffer was added and vortexed prior to acquiring the results on flow cytometer.

CAR gene sequence:

The amino acid sequence of the IL-15R α CD19 CAR tested in this study is as follows, broken down by the domains included in the CAR:

DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGV
PSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGSTSGSGKPG
SGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWL
VIWGSETTYNSALKSRLTIHKDNSKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYA
MDYWGQGTSVTVSSAA (CD19 scFv domain)

IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFPGPSKPFWVLVVVGVLACYSLLVT
VAFIIFWVRSKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSAS (CD28
transmembrane/cytoplasmic domain)

GGGGSGGGGS (linker)

KSRQTPPLASVEMEAMEALPVTWGTSSRDEDLENC SHHL (IL-15R α cytoplasmic
5 signal domain)

GGGGSGGGGS (linker)

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
10 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
PR (CD3zeta signal domain) (SEQ ID NO: 31)

RESULTS

Construction of CARs containing various co-stimulatory signaling domains

15 The CD19 CAR (19z) sequence was codon optimized and chemically synthesized based on the anti-CD19 scFv of mouse hybridoma FMC63 {Nicholson, 1997, Construction and characterization of a functional CD19 specific single chain Fv fragment for immunotherapy of B lineage leukaemia and lymphoma}. Briefly, the parental 19z CAR (3rd generation) contained a chimeric intracellular signaling element derived from CD28 trans-
20 membrane and cytoplasmic domain, the co-stimulatory 4-1BB intracellular TRAF binding domain and the CD3 ζ chain intracellular domain, analogous to the CAR design of Kochenderfer et al. {Kochenderfer, 2009, Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor}. To establish new generation CARs, the co-stimulatory domains of various immune modulatory receptors were incorporated into the CAR construct
25 including the cytoplasmic domains of CD27 (273z), OX40 (OX40z), ICOS (ICOSz), and IL-15R α (153z). These CARs were cloned into the lentiviral vector pTYF and packaged with NHP/VSVG lentivector system as illustrated in FIG. 1.

Development of a simplified killing assay for functional evaluation of CARs

To demonstrate CAR-mediated specific killing of target cells, a simplified killing

assay was developed based on CAR-modified Jurkat T cells and target cells that were genetically engineered to express a green fluorescent protein (wasabi) or labeled with calcein AM. To illustrate CART-mediated target killing, the Jurkat-CARTs (effectors) were incubated with the GFP-labeled target at different effector:target ratios, and at various time points, the cultured cells were stained with annexin V and PI and analyzed by flow cytometry. The diagram in FIG. 2 illustrates the killing assay; representative flow cytometry graphs demonstrate the ratio change of the green target cells (%), the altered target cell morphology shown by increased side scatter (SSC) reflecting apoptosis, and the populations of early and late apoptotic cells (annexin V-stained) and dead cells (PI-stained). All CAR constructs were verified for specific target killing activities prior to the primary T cell gene transfer studies.

Evaluation of CART killing efficiency using primary T cells

Jurkat CARTs are convenient for short term killing assessment. However, Jurkat CARTs lack long term killing function due to high PD-1 up-regulation after target engagement. Therefore, T cells from healthy donors were transduced with lenti-CAR vectors and tested for CART target killing activities. The efficiency of lentivector modification of primary T cells is very high (FIG. 3). Healthy donor T cells were transduced with 19z, 273z OX40z, ICOSz, and 153z lenti-CAR vectors. As control, GD2-CARTs expressing the ganglioside antigen GD2-specific CAR were included. RS4;11w, a CD19+ve B-ALL cell line expressing wasabi GFP gene, was used as a specific target. Both target and effector cells were co-cultured in a ratio of 1:2 in a 96 U shaped well plate. Killing was assessed at 24-48 hr and pictures were obtained under fluorescent microscope. Representative results are illustrated in FIG. 4, which shows almost complete disappearance of the green target cells in the 153z coculture well, and decreased target in 19z, 273z, OX40z and ICOSz groups compared to target only and the non-specific GD2-CART groups. Repeated assays showed consistently that 153z had a rapid killing kinetics compared to the others.

Highly efficient and rapid target killing kinetics of 153z CAR in both healthy donor and leukemic patient T cells

To further evaluate the rapid target killing activities of 153z CAR, the RS4;11w target was cocultured with healthy donor PBMC-derived 19z, 273z, 153z and control GD2z CARTs at 1:1 ratio for one hour, and cells were stained with annexin V and PI and analyzed by flow cytometry. The result confirmed that 153z CARTs killed the target at the highest efficiency quickly based on the analysis of early apoptotic cells by annexin V staining (annexin V positive and PI negative, FIG. 5A). This assay was repeated in triplicates and both percentages of total cell death and specific target lysis were analyzed, and statistical significance was obtained as shown in FIG. 5B.

To examine the activities of 153z CAR in leukemic patients' T cells, RS4;11 was a target killing assay was performed using CARTs generated from B-ALL patients' T cells. 19z, 273z and 153z CARs were compared in three leukemia patients' T cells in a short term assay as described above. The results again confirmed that 153z displayed the fastest killing kinetics as illustrated by annexin V staining for early apoptotic cells in 1-2 hours (FIG. 6A). This was evident after 1-3 day coculture when cell death was analyzed by annexin V/PI staining and the disappearance of GFP+ target cells examined by flow cytometry and under fluorescent microscope (FIGs. 6B, 6C).

Effective killing of patient leukemic cells by autologous 153z CARTs

The above assays used B-ALL cell line RS4;11 as target cells. To illustrate killing of the patients' own leukemic cells, B-ALL patients' leukemic cells were obtained from bone marrow of two patients. These cells were labeled with green fluorescent dye calcein AM and co-cultured with autologous CARTs of 19z, 273z and 153z from the two B-ALL patients at effector:target ratio of 1:5. Target cell death was examined after 1 hour and 1 day. FIG. 7 demonstrates that 153z CARTs displayed the same trend of high target killing activities as compared with 19z and 273z in 1 hr (FIG. 7A) and 1 day (FIG. 7B) co-cultures of patients' own leukemic cells. Similar results were obtained for the second patient (not shown).

Prolonged killing function of 153z CARTs

Next it was determined if these CD19 CARTs retained their ability to kill more target cells after they had already been exposed to target once. After the green target was completely gone, the remaining live cells were counted, which were mainly the CART

effector cells. An equal number of the various CARTs was added to 10 times RS4;11w target in further rounds of killing, and flow cytometry was performed to determine the green target cells left at various time points. Both a healthy donor and a leukemic patient CARTs were tested. The results showed that target was eliminated efficiently after even fourth rounds of coculture, all within 1-2 days, as shown with the leukemia patient CARTs in FIG. 8. Importantly, 153z, although killed rapidly, retained capacity to further kill more target cells.

Increased T cell proliferation and effector functions of 153z CAR

The above results indicate that 153z had high target killing activities compared to 19z and 273z. Without wishing to be bound by theory, the target killing function of the CAR should be correlated with intracellular signaling for T cell proliferation and effector cytokine release. Next, T cell proliferation assays were performed based on CFSE labeling of T cells. Control T cells or CARTs were labeled with CFSE and cocultured with leukemic target cells for 3 days. Flow cytometry analysis revealed that 153z CARTs displayed the highest proliferation rate as compared with 19z and 273z CARTs (FIG. 9). For the effector function analysis, CARTs were stained for intracellular IL-2, IFN- γ and degranulated CD107a after incubation with target cells. The results illustrated that 153z CARTs displayed the highest effector cytokine expression when co-cultured with target (FIGs. 10A-10C). To confirm the intracellular cytokine results, a flow cytometry-based cytokine bead assay (CBA) was used to determine the concentration of various cytokines secreted into the cocultures. In this assay, T cells without CAR and with no target added and T cells stimulated with PMA and ionomycin were included as negative and positive controls, respectively. Supernatants from 153z CART co-culture collected on 48 hr contained the highest amount of IFN- γ and IL-2 compared to 19z and 273z CART co-cultures.

Genetically modified T cells have shown great promise in the treatment of B cell malignancies in the last five years. Among a number of published CART trials in hematological malignancies, CD19 or CD20 has been an effective target {Porter DL, 2011, Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia.; Brentjens RJ, 2013, CD19-targeted T cells rapidly induce molecular remissions in adults with

chemotherapy-refractory acute lymphoblastic leukemia.; Kochenderfer JN, 2013, Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation.; Grupp SA, 2013, Chimeric Antigen Receptor-Modified T cells for Acute Lymphoid Leukemia.}. Several variables are still being

5 investigated in determining the CART efficacy and persistence *in vivo*. One of the important determinants of CART efficacy is the choice of activation or co-stimulatory signals in the CAR structure. The study described herein made an attempt to further perfect the CAR design by exploring the potent activating signal of IL-15R α (153z CAR) as a co-stimulatory domain and found that these CARTs have rapid reaction kinetics and high targeting efficacy.

10 To assess CAR functions, an efficient lentiviral vector gene delivery system has been applied and a rapid cytotoxic assay without pre-selection of CAR transduced T cells has been developed. The different CARTs were transduced with lenti-CAR vectors at similar efficiencies based on quantitative PCR confirming the CAR transgene copy number in the cells. The 153z CARTs killed target CD19+ cells very rapidly as compared to 19z and 273z

15 CARTs in short term assays from 30 min to two days. To assess long term killing effects, more than ten times of target cells were repetitively added to the CARTs, and interestingly, the CARTs became more effective after the first round of killing, likely due to selective expansion of functional CARTs. The latter is similar to the findings of Henderson et al. who has reported enhanced killing of CARTs on re-encountering the target{Henderson, 2013,

20 Chimeric antigen receptor-redirected T cells display multifunctional capacity and enhanced tumor-specific cytokine secretion upon secondary ligation of chimeric receptor}. This is also consistent with *in vivo* observation that infused CARTs can expand more than 1,000 fold in leukemia patients{Porter DL, 2011, Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia.; Brentjens RJ, 2013, CD19-targeted T cells rapidly induce molecular

25 remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia.}.

The assessment was further conducted using the primary patient T cells with CAR and patient ALL cancer cells. A much higher ratio of target:effector such as 10:1 was tested to closely parallel the *in vivo* situation where tumor burden may be much higher compared to the infused CART cells. Effective killing was observed even in this setting. These results

indicate that the CD19-specific CART cells can efficiently target ALL coupled with high proliferation rate, activation of effector functions, resulting in complete eradication of cancer cells. The CFSE-based proliferation assay indicates that the 153z CARTs proliferated to the greatest extent compared to the 19z and 273z CARTs. One of the factors contributing to the enhanced killing seen with 153z CARTs might be the rapid expansion of these cells when they see target. In addition, 153z CARTs secreted increased amount of effector cytokines such as IFN- γ , TNF α and IL-2 upon target engagement, as compared with 19z and 273z CARTs. Therefore, without wishing to be bound by theory, the enhanced target killing of 153z CARTs is conceivably a result of both rapid response kinetics and high effector activities.

As with T cell nature and biology, expansion and differentiation could result in exhaustion. 153z cells killed more rapidly and efficiently when they encountered target, so they could exhaust rapidly as well. Re-targeting experiments were performed by counting the cells from the co-culture after all the target was gone and adding more target in a ratio of 10:1 target to effector to a 48 well plate. This retargeting demonstrated that the target was killed more rapidly as the T cells were already in an activated, effector state. The 153z cells retained their capacity to kill as effectively as the 19z and 273z cells in the retargeting experiment. Interestingly, the increased activated state of 153z CART was associated with decreased PD-1 expression on these cells after the first round killing, suggesting that initial expansion of 153z CARTs is not very exhaustive.

Without wishing to be bound by theory, when CART binds the tumor antigen, this sends an activation signal and converts the memory T cells into effector T cells which mediates the killing by perforin-granzyme pathway, and the release of effector cytokines leading to sustained T cell activation{Trapani, 2002, Functional significance of the perforin/granzyme cell death pathway}. CD107a degranulation is another marker for T cell effector function associated with cytolytic activities{Betts, 2004, Detection of T-cell degranulation: CD107a and b. Methods in Molecular Medicine}. The study herein showed the differential production of these cytokines and up-regulation of CD107a expression when the different CART cells were encountering target by intracellular cytokine staining, and

confirmed that these effector markers were in line with the strength of the T cell activation signal and target killing function. Again, the highest effector markers were observed with 153z CARTs.

In a similar study, Hoyos et al. have reported CARTs with CD28 co-stimulatory domain along with IL-15 gene and an inducible caspase gene, which ectopically over express IL-15 to enhance killing, which could potentially lead to excessive IL-15 production which is known to be a tumor growth cytokine{Hoyos, 2010, Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety}. In 153z CAR, the IL-15R α cytoplasmic domain is engineered in tandem with CD28 and CD3 ζ , which confers a stimulating effect only when the CARTs encounter target. Thus, this design endows IL-15 signaling in the CARTs only independent of extracellular IL-15 expression.

In summary, the IL-15R α endodomain confers a potent stimulus to the CART when it encounters target, leading to enhanced proliferation, effector activities and target killing. In clinical settings, during the initial treatment when disease burden is high, CARTs with rapid killing efficacy could be advantageous and once this effect is seen, maintenance CARTs may be applied for persistence. Besides leukemia, it is also anticipated that the rapid 153z CAR effector kinetics may achieve better therapeutic outcomes in the more difficult to target tumors such as solid tumors.

Example 2: Demonstration of safety and efficacy of chimeric antigen receptor (CAR)-modified T cells for the treatment of relapsed or refractory CD30 positive lymphomas

The leukocyte activation marker CD30 (TNFRSF8) is a 120 kDa type I transmembrane cytokine receptor from the tumor necrosis factor receptor (TNFR) superfamily. CD30 is consistently expressed in Hodgkin lymphoma (HL), anaplastic large cell lymphoma (ALCL), and variably expressed in other B and T cell lymphomas, including diffuse large B-cell lymphoma (DLBCL), primary effusion lymphoma, adult T-cell leukemia/lymphoma, mycosis fungoides, and extranodal natural killer/T-cell lymphoma. A large number of CD30 positive lymphoma patients cannot be cured by standard chemo-

radiotherapy. Brentuximab Vedotin (SGN-35) is an antibody-drug conjugate directed against the CD30 antigen expressed on lymphoma cells, which has been approved by U.S. Food and Drug Administration (FDA) for the treatment of relapsed or refractory classical HL and systemic ALCL. However, SGN-35 is not available or approved in many countries.

5 Therefore, although CD30 represents an attractive target for immunotherapy, there remains a need to develop alternative CD30-targeting therapeutics.

Described in this example are a experiments related to a chimeric antigen receptor (CAR) that targets CD30 and a clinical trial of autologous T cells expressing this CAR. Pre-clinical study results and the preliminary outcome of one patient enrolled in a clinical trial of anti-
10 CD30 CAR T cells for the management of relapsed and refractory CD30 positive lymphomas (www.clinicaltrials.gov; #NCT02274584) are reported.

MATERIALS AND METHODS

Cell lines and media

15 CD30-positive target cells were used for the CAR T cell target killing assays. Primary CD30+ ALCL cells and B lymphoma cells were established in the laboratory. These cells were maintained in RPMI1640 medium (Life Technologies, Inc. Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc. Norcross, GA), penicillin (100 units/ml) and streptomycin (100ug/ml). Target cell lines were transduced
20 with lentiviral vectors expressing a green fluorescent protein (wasabi GFP) and the reporter gene positive cells were sorted by flow cytometry (see FIG. 11). T cells were transduced with lentiviral CD30 CAR or control CAR vectors and the copy number of CAR per cell was determined by quantitative PCR using genomic DNA.

Blood donors and primary T cell culture

25 Buffy coats from healthy donors (HDs) were purchased from LifeSouth Civitan Blood Center (Gainesville, FL, USA) with approval of Institutional Review Board (IRB-01) of University of Florida. PBMCs were isolated from buffy coats by gradient density centrifugation in Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Piscataway, NJ, USA). Blood and bone marrow samples of Hodgkin's lymphoma patients were obtained from

Peking University Cancer Hospital with IRB approved protocol. T cells were activated using anti-CD3 and anti-CD28 antibodies or phytohemagglutinin (PHA). The T cells were maintained in TexMACS medium (Miltenyi Biotec Inc, San Diego, CA) supplemented with interleukin-2, -7 and -15. Phenotype analysis of the activated cells by flow cytometry was performed to confirm T cell purity. After expansion for two to six days, the T cells were transduced with lentiviral CAR vectors.

CD30 CAR synthesis, lentiviral vector construction and CAR gene transduction

The CD30 CAR sequence was codon optimized and chemically synthesized based on two published mAb clones, AC10 (a murine hybridoma IgG2b clone, J. Immunol. 1993, Dec 1; 151(11): 5896-5906), and a humanized mAb clone 5F11. The amino acid sequences of the two scFv clones used for CD30 CAR engineering are listed below.

AC10 scFv:

VH

QIQLVQSGAEVKKPGASVKVSCAS

GYTFTDYYIT (CDR1)

WVRQAPGQGLEWMG

WIYPGSGNTKYNEKFKG (CDR2)

RVTMTRDTSISTAYMELSRLLRSDDTAVYYCAN

YGNYWFAY (CDR3)

WGQG TLVTVSS (SEQ ID NO: 14)

VL:

DIVMTQSPDSLAVSLGERATINC

KASQSVDFDGD SYMN (CDR1)

WYQQKPGQPPLLIY

AASNLES (CDR2)

GVPDRFSGSGGTDFLTISLQAEDVAVYYC

QQSNEDPWT (CDR3)

FGQGTKVEIK (SEQ ID NO: 13)

5F11 CD30 scFv:

VH

5 QVQLQQWGAGLLKPSETLSLTCAVYGGSF
 AYYWS (CDR1)
 WIRQPPGKGLEWIG
 DINHGGGTNYNPSLKS (CDR2)
 RVTISVDTSKNQFSLKLNSVTAADTAVYYCAS
 10 LTAY (CDR3)
 WGQGS�VTVSS (SEQ ID NO: 16)

VL

DIQMTQSPTSLSASVGDRVITC
 15 RASQGISSWLT (CDR1)
 WYQQKPEKAPKSLIY
 AASSLQS (CDR2)
 GVPSRFSGSGSGTDFTLTISLQPEDFATYYC
 QQYDSYPIT (CDR3)
 20 FGQGTRLEIK (SEQ ID NO: 15)

The CAR contains a chimeric intracellular signaling element derived from CD28 trans-membrane and cytoplasmic domain, the co-stimulatory 4-1BB intracellular TRAF binding domain, a CD27 cytoplasmic domain, and the CD3 ζ chain intracellular domain. The
 25 CAR gene was cloned into the lentiviral vector pTYF and packaged with NHP/VSVG lentivector system, as shown in FIG. 12.

Lentiviral vectors were generated based on the NHP/TYF lentiviral vector system. CAR DNA was chemically synthesized and cloned into pTYF transducing vector behind human EF1 α promoter. The final lentiviral-CAR constructs were verified by restriction

enzyme mapping and DNA sequencing.

CAR gene sequence

The amino acid sequence of the CD30 CAR tested in this study is shown as follows, broken down by the domains included in the CAR. A schematic of a CD30 CAR is shown in FIG. 13:

QVQLQQWGAGLLKPSETLSLTCAVYGGSFSAYYWSWIRQPPGKGLEWIGDINHGGG
TNYNPSLKSRTISVDTSKNQFSLKLNSVTAADTAVYYCASLTAYWGQGSLVTVSS
(CD30 scFv domain)

GSTSGSGKPGSSEGSTKG (Linker)

DIQMTQSPTSLASVGDRVTITCRASQGISSWLTWYQQKPEKAPKSLIYAASSLQSGV
PSRFSGSGSGTDFTLTISLQPEDFATYYCQQYDSYPITFGQGTRLEIK (CD30 scFv
domain)

GSTSGSGKPGSSEGSTKG (Linker)

FWVLVVVGGVLACYSLVTVAFIIFWV (CD28 transmembrane domain)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL (4-1BB intracellular
domain)

QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (CD27
intracellular domain)

GSTSGSGKPGSSEGSTKG (Linker)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDRRGRDPKRRKNPQ
EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMALP
PR (CD3 zeta domain)

GSTSGSGKPGSSEGSTKG (Linker)

VGALESLRGNADLAYILSMPCGHCLIINNPNFCRESGLRTRTGSNIDCEKLRRRFSSL
HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDSPGSN
PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (truncated
iCasp9 domain)

MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFGMLGKQE
 VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE (FKBP
 domain) (SEQ ID NO: 32)

In some embodiments, the above exemplary, non-limiting arrangements are from left to right,
 5 N-terminus to C-terminus of the CAR. The CAR may comprise or further comprise any
 other combination of elements as described herein.

CAR detection by PCR

The CAR transgene copy numbers in CART cells were determined by quantitative
 SYBR green real time PCR (qRT-PCR). Genomic DNA was harvested from CART cells
 10 using Promega Wizard genomic DNA purification kit (Promega Corp. Madison, WI). The
 qRT-PCR reaction condition was as suggested by SABioscience and data collected using
 MX3000P (Stratagene, Agilent Technologies, Santa Clara, CA).

Calcein AM labeling of primary tumor cells

Fresh or thawed tumor cells were first washed with RPMI1640 medium. Then 1×10^6
 15 cells were suspended in 196 μ l of RPMI1640 in a tube, and 4 μ l of Calcein AM (Life
 Technologies Corp., 20 μ M) was added to get a final concentration of 0.4 μ M. The cells were
 incubated at 37°C for 1 hour and washed twice with RPMI containing fetal bovine serum
 before used in co-culture experiments.

CAR T cell killing assay

20 Target cells and CAR T cells were counted using trypan blue staining and a
 hemocytometer. The cells were co-cultured in 96U plate in effector:target ratios of 1-2:1, or
 as indicated in each experiment. CAR T cell numbers ranged from 5×10^4 to 2×10^5 . The
 coculture was incubated in TexMACS medium without cytokines. Cells were incubated for
 1-2 hours or overnight for short term assays. CAR T-targeted killing was recorded by
 25 quantitative analysis of the shifted side scattered (SSC) population, and early and late
 apoptotic (annexin V-stained) and Propidium Iodide (PI)-stained (dead) cells. Samples were
 run in the LSRII flow cytometer using the DiVa software (BD Biosciences) and results were
 analyzed using the FlowJo software. An example of results obtained using this assay is
 shown in FIG. 14.

Annexin V and PI staining for apoptosis

At different time points after incubation, cells were transferred to 96-V plate and centrifuged at 400g for 5 minutes to remove the media. Then the cell pellet was washed with PBS once. Cells were re-suspended in 30μl of staining mix consisting of 0.25μl PI (Sigma), 0.5μl annexin V (BD Bioscience) and 29.25μl of binding buffer (BD Bioscience), and then incubated in the dark for 15 minutes at room temperature. Subsequently, cells were suspended in 200μl FACS buffer and analyzed in the LSRII flow cytometer using DiVa software (BD Biosciences). Results were evaluated using FlowJo software.

Monoclonal antibodies

Fluorochrome-conjugated monoclonal antibodies against human IFN-γ (clone B27, APC), IL-2 (clone MQ1-17H12, PE) CD8 (clone SK1, APC-Cy7), CD4 (clone RPA-T4, PB), CD30 (clone 50614, FITC), CD27 (clone M-T271, APC), CD 28 (clone L-293, PerCp-Cy5.5), CD34 (clone 8G12, PE-Cy7), PD1 (clone EH12.1, PE-Cy7), CD107a (clone H4A3, FITC) were purchased from BD Biosciences (San Diego, CA). Anti-CD19 (clone SJ25C1, PE) and anti-CD127 (clone eBio-RDR5, APC-eflour) antibodies were purchased from Caltag Laboratories (Life technologies Inc) and eBiosciences, respectively.

Surface staining and intracellular cytokine staining of CAR T cells

For effector functional analysis, the CART cells were mixed with target cells in an E:T ratio of 1:3 overnight with the addition of 1.5μl of FITC-conjugated anti-CD107a Ab. Positive control used T cells (without CAR) stimulated with PMA (1μg/μl) and Ionomycin (1ng/μl) for 1 hour. The intracellular cytokines were immobilized after treated with monensin (6μg/μl) for 6 hours. The samples were then washed, blocked with 10% human and mouse sera for 30 min, stained with anti-CD4 and anti-CD8 Abs for 30 min, fixed and permeabilized with BD Fix/Perm Buffer, stained with anti- IFN-γ and anti-IL-2 Abs for 1 hour, and then analyzed by flow cytometry. Data was collected on the BD LSRII flow cytometer and analyzed with FlowJo.

RESULTS

The patient is a 22 year-old male. He was diagnosed with stage III Hodgkin's lymphoma (Nodular Sclerosis), and was treated with six cycles of ABVD and radiation to the

residual disease. The response to this treatment was complete remission. Subsequently, the disease relapsed confirmed by biopsy of the left supraclavicular lymphadenopathy. After two cycles of COEP, disease kept progressing. The patient then received two cycles of ICE followed by partial remission. The patient was later treated with auto-transplant. This treatment resulted in complete remission. The disease relapsed again, with nodules in lung and mediastinal, abdominal, retroperitoneal lymphadenopathy. Subsequently, the patient was enrolled in a clinical trial of anti-CD30 chimeric antigen receptor-modified T cells.

Detection of CAR T cells in vitro

FIG. 15 shows a fluorescent image of target killing assay of two different CD30-CAR T cells based on 5F11 and AC10 scFv CD30-CARs. The primary lymphoma cell line was labeled with green fluorescent protein and used as target cells. CAR T cells and target cells were coincubated in a 96 U shaped well at 1:1 ratio for 12 days. The disappearance of green fluorescent indicates target cell killing by CAR T cells. The AC10 CD30-CAR displayed the best target killing activity.

FIG. 16 shows flow cytometry target killing assay of two different CD30-CAR T cells based on 5F11 and AC10 scFv CD30-CARs. The primary lymphoma cell line was labeled with green fluorescent protein and used as target cells. CAR T cells and target cells were coincubated in a 96 U shaped well at 1:1 ratio for 12 days. The disappearance of green fluorescent cells detected by flow cytometry indicates target cell killing by CAR T cells. The AC10 CD30-CAR displayed the best target killing activity.

Detection of CAR T cells in vivo

Quantitative polymerase chain reaction was performed to detect CAR T cells in blood. The CD30-CAR T cells were detected on day 14 after first infusion. On day 45, CAR T cells reached its peak level accounting for more than 20% of circulating lymphocytes. This peak level of CAR T cells coincided with the observed disease remission and results of serum cytokine levels. CAR T cells decreased 80 days after cell infusion, which paralleled with disease progression. Interestingly, CAR T cells increased to ~8% again on day 140 indicating *in vivo* response to tumor relapse (FIG. 17).

Detection of cytokine response after CAR T infusion

Serum cytokines were measured and showed evidence of immune activation. The patient's disease remission was accompanied by an increase in levels of inflammatory cytokines, with levels of interferon- γ and interleukin-6 peaking around 40 days after first cell infusion. The increase in serum cytokines paralleled with disease remission (FIGs. 18-19).

Example 3. Chimeric antigen receptor expressing T cells mediate activity against osteosarcomas

Since the prognosis for children with high risk osteosarcoma (OS) remains suboptimal despite intensive multi-modality therapies, there is a clear and urgent need for the development of targeted therapeutics against these refractory malignancies. Chimeric antigen receptor (CAR) modified T cells can meet this need by utilizing the immune system's surveillance capacity and potent cytotoxic mechanisms against tumor specific antigen targets with exquisite specificity. Since OS highly expresses the GD2 antigen, a viable immunotherapeutic target, the study described herein sought to assess if CAR modified T cells targeting GD2 could induce cytotoxicity against OS tumor cell lines. It was demonstrated that the OS cell lines U2OS, HOS, and a primary human OS cell line highly express the GD2 antigen (>80%), and that GD2 CAR modified T cells were highly efficacious for inducing tumor cell death. Interestingly, the OS cells were induced to up-regulate expression of PD-L1 upon interaction with GD2 CAR modified T cells, and the specific interaction induced CAR T cells to overexpress the exhaustion marker PD-1 along with increased CAR T cell apoptosis. To further potentiate CAR T cell killing activity against OS, it was demonstrated that checkpoint blockade along the PD-L1/PD-1 axis can synergize with CAR T cell therapy. In addition, conventional chemotherapy in combination with CAR T cell therapy can also synergize the effects showing an increased target cell killing activities.

MATERIALS AND METHODS

Cell lines

HOS and U2OS cells were cultured at 37 °C in 4% CO₂ atmosphere in Dulbecco's

modified eagle medium; DMEM (GIBCO®, Life technologies, CA, USA) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin.

Jurkat cells were cultured at 37 °C in 4% CO₂ atmosphere in RPMI-1640 medium (GIBCO, Life Technologies) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin.

Detection of GD2 surface expression

The ~80% confluent cells were harvested using 2.5 mM EDTA for HOS and 5 mM EDTA for U2OS. The cells were sampled, mixed with trypan blue and counted. For GD2 surface staining, 10⁵ cells were collected and washed twice with PBS and blocked with 10% human-mouse (1:1) serum in FACS buffer (2% FBS, 0.1% NaN₃ in PBS) at 4 °C for 30 minutes. The cells were washed with FACS buffer and stained with PE conjugated anti-GD2 Ab (BD biosciences, CA, USA) and kept in the dark at 4 °C for 30 minutes. The cells were washed with FACS buffer and fixed with 1% paraformaldehyde in PBS. GD2 surface expression were detected by BD LSRII flow cytometer and analyzed with FlowJo.

Percentage of GD2 expression and MFI were determined by subtraction background obtained from isotype control (PE conjugated mouse IgG2a, BD Pharmingen, CA, USA).

Construction of lentiviral vectors

Lentiviral vectors were generated using the NHP/TYF lentiviral vector system as previously described [16, 17]. CAR DNA was chemically synthesized and cloned into pTYF transducing vector behind human EF1 α promoter using standard molecular cloning approaches. The final lentiviral- constructs were verified by restriction enzyme mapping and DNA sequencing.

Blood donors, PBMC isolation and T cell activation

Blood samples were obtained from a donation center or osteosarcoma patients. PBMC were isolated using Ficoll-Paque plus (GE Healthcare). T cells were activated using anti-CD3 and anti-CD28 antibody-conjugated magnetic beads or phytohemagglutinin (PHA). The T cells were maintained in TexMACS (Miltenyi Biotec Inc, San Diego, CA) or AIM-V (Invitrogen) supplemented with interleukin-2, -7 and -15 as previously described [18]. Phenotype analysis of the activated cells were verified to confirm T cell purity. After

expansion for two to six days, the T cells were transduced with lentiviral CAR vectors and evaluated killing function.

GD2-CAR-T cell cytotoxicity analysis

GFP-positive OS cells were prepared as single cell suspension as mentioned above.

5 The cells were resuspended in DMEM growth medium at 1.5×10^5 cells/ml and 200 μ l of the OS cells were seeded into 48-well plate and cultured at 37 °C in 5% CO₂ for two hours.

CAR-modified T cells (primary T cells or Jurkat cells) were added to the wells of OS cells at various effector to target (E:T) ratio and co-cultured for various period of time in the present or absent of anti-PD-L1 antibody (BioLegend, CA, USA). The cells were monitored

10 periodically under fluorescence microscope (Zeiss Axiovert 25) and photographed.

For flow cytometer analysis of cell death, the cells were harvested using EDTA as described.

The harvested cells were washed once with PBS and stained with Annexin V-V450 (BD Biosciences) and propidium iodide (Sigma) for 10 minutes at room temperature. After

staining, the cells were resuspended in 1% paraformaldehyde in PBS. Data were collected by

15 BD LSRII flow cytometer and analyzed with FlowJo. The difference of cell death between GD2 CAR-modified T cells and control-CAR T cells was analyzed by independent t-test with P value ≤ 0.05 being significant. Percent specific lysis of target cells was calculated based on the following formulation [19]:

20
$$\% \text{ specific lysis} = (\% \text{ apoptosis of target cell} - \% \text{ spontaneous cell apoptosis}) /$$
$$(100\% - \% \text{ spontaneous cell apoptosis}) \times 100$$

PD-1 and PD-L1 surface staining of the co-cultured cells

25 The co-cultured OS cells and CAR-modified T cells were harvested and blocked with 10% human-mouse (1:1) serum at 4 °C for 30 minutes. The cells were washed and stained with PE-Cy7 conjugated anti-PD-L1 (eBiosciences, CA, USA) or PE-Cy7 conjugated anti-PD-1 (BD Biosciences) at 4 °C for 30 minutes in the dark. The cells were washed with FACS buffer and fixed with 1% paraformaldehyde in PBS. PD-1 or PD-L1 surface expression was analyzed using BD LSRII flow cytometer and FlowJo software. For analysis of PD-1

expression in CAR-modified T cells, the FITC negative T cells were gated while for PD-L1 expression the FITC positive cells were gated. Percentage of PD-1 or PD-L1 expression and mean fluorescence index (MFI) were determined by subtraction of background isotype control (PE-Cy7 conjugated mouse IgG1 κ ; BD Biosciences).

5

RESULTS

Analysis of GD2 surface expression in OS cell lines.

Two OS cell lines including HOS and U2OS cells were analyzed for expression of GD2 by flow cytometry. GD2 expression was found on both OS cell lines, 80.1% and 99% on U2OS and HOS cells, with MFI of 7,066 and 26,796, respectively (FIG. 20). In a primary OS cell culture, OS156, a high level of GD2 expression was detected (94.3%, MFI=72,080, FIG. 20).

10

Construction of 4th generation GD2 CAR lentiviral vectors.

To engineer GD2-specific CARs, three humanized GD2-specific scFv clones, hu3F8, c.60C3 and hu14.18[15, 20, 21] were selected. These GD2 CAR sequences were then human codon-optimized and chemically synthesized. To establish 4th generation CARs, several intracellular T cell signaling motifs were incorporated in the CARs including CD28 transmembrane and cytoplasmic domain, the co-stimulatory 4-1BB intracellular TRAF binding domain, the CD27 cytoplasmic domain, and the CD3 ζ chain intracellular domain as illustrated in FIG. 21. These CAR genes were cloned into the lentiviral vector pTYF and packaged into lentiviral particles for gene transfer.

15

20

hu3F8 scFv: (VH and VL linked by 218S linker which is underlined)

QVQLVESGPGVVQPGRSLRISCAVSGFSVTNYGVHWVRQPPGKGLEWLGVIWAGGI
 TNYNSAFMSRLTISKDNSKNTVY^{218S}LQMNSLRAEDTAMYYCASRGGHYGYALDYWG
 QGTLVTVSSGSTSGSGKPGSSEGSTKGEIVMTQTPATLSVSAGERVTITCKASQSVSN
 DVTWYQQKPGQAPRLLIYSASNRYSGVPARFSGSGYGTEFTFTISSVQSEDFAVYFCQ
 QDYSSFGQGTKLEIK (SEQ ID NO: 19)

25

C60c3 ScFv: (VH and VL linked by 218S linker which is underlined)

EVKLVESGGGLVLPGLSLRLSCATSEFTFTDYMTWVRQPPRKALEWLGFIRNRAN
 GYTTEYNPSVKGRFTISRDNQSILYLQMNTLRTEDSATYYCARVSNWAFDYWGQG
 TTLTVSSGSTSGSGKPGSSEGSTKGDVVMQTQPLSLPVSLGDQASISCRSSQSLLKNN
 5 GNTFLHWYLQKSGQSPKLLIYKVSNRLSGVPDRFSGSGSGTYFTLKISRVEAEDLGV
 YFCSQSTHIPYTFGGGTKLEIK (SEQ ID NO: 20)

Hu14.18 scFv: (VH and VL linked by 218S linker which is underlined)

EVQLVQSGAEVEKPGASVKISCKASGSSFTGYNMNWVRQNIGKSLEWIGAIIDPYYG
 10 GTSYNQKFKGRATLTVDKSTSTAYMHLKSLRSEDVAVYYCVSGMEYWGQGTSTTV
 SSGSTSGSGKPGSSEGSTKGDVVMQTQPLSLPVTPGEPASISCRSSQSLVHRNGNTYL
 HWYLQKPGQSPKLLIHKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQS
 THVPPLTFGAGTKLELK (SEQ ID NO: 21)

15 The above scFv domains were linked to the following CAR-inducible caspase 9 sequence
 (domain names are listed after each domain in bold);

FWVLVVVGGLVACYSLLVTVAFIIFWV (**CD28 transmembrane domain**)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL(**4-1BB intracellular
 domain**)

20 QRRKYRSNKGESPEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (**CD27
 intracellular domain**)

GSTSGSGKPGSSEGSTKG(**218S linker**)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKPRRKNPQ
 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP

25 PR (**CD3zeta**)

GSTSGSGKPGSSEGSTKG (**218S linker**)

VGALESRLGNADLAYILSMPCGHCLIINNPNFCRESGLRTRTGSNIDCEKLRRRFSSL
 HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
 TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDESPGSN

PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
 FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (**truncated
 Casp9**)
 MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFFKMLGKQE
 5 VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE
 (**FKBP domain**) (SEQ ID NO: 37)

Functional evaluation of GD2-CARs based on a rapid killing assay.

A simple and quick killing assay was developed to demonstrate GD2-CAR-mediated
 10 specific killing of OS cells. The assay was based on co-incubation of CAR-modified T cells
 and green fluorescent (wasabi GFP) GD2-positive OS cells. CAR T cells (effectors) were
 incubated with green target cells at different effector/target ratios, and at various time points,
 the cultured cells were analyzed by flow cytometry after staining with annexin V and
 propidium iodide (PI). FIG. 22 demonstrates the killing assay; the target cell death is
 15 detected by disappearance of green cells shown by change in E/T ratios and increased
 apoptotic and PI-stained dead cells. The hu3F8 CAR was chosen as the focus for the
 following experiments.

GD2-CAR-T cell cytotoxicity against OS cell lines.

To demonstrate specific OS killing, the target U2OS and HOS cells were transduced
 20 with a lentiviral green fluorescence wasabi gene to generate U2OSw and HOSw reporter cell
 lines. The percent specific lysis was assessed for early and late apoptosis by flow cytometric
 analysis of Annexin V and PI. In a simplified assay, JK cells were transduced with GD2-
 CAR or control CD19-CAR and cocultured with U2OSw or HOSw to assess the CAR-T
 killing ability. Compared with control (non-specific) CD19-CAR-JK cell, the percent
 25 specific lysis by GD2 CAR-JK cells was significantly increased against U2OSw (FIGs. 23A,
 B; $p = 0.0049$). Similar trend was observed against HOSw (FIGs. 23C, 23D, $p = 0.078$).

Primary GD2 CAR T cells targeting established and primary OS cell lines.

Primary T cells from healthy donors were transduced with GD2 CAR and co-cultured
 with OS cell lines U2OSw and HOSw in a series of effector to target ratios (4:1, 2:1, 1:1 and

1:2), and the cells were harvested and evaluated after 1 day. Compared with control T cells and/or non-specific CAR (CD19-CAR), percent specific lysis of U2OSw was increased when co-cultured with GD2 CAR T cells but not with non-specific control CD19-CAR T cells (FIGs. 24A, 24B). Similar results were obtained with HOSw cells using GD2-CAR T cells but not another non-specific glypican 3 (GPC3)-CAR T cells (FIGs. 24C, 24D). To see if the OS target killing effects could be detected with primary tumor cell line, the OS156 cells, established from an OS patient, was tested for GD2-CAR T killing assay. A green fluorescent wasabi gene modified OS156 cell line, OS156w, was generated and used as target cells. After 1 day co-cultured at E:T ratio 1:1, GD2 CAR-T cells also efficiently killed the primary OS156w cells as illustrated by specific lysis of target cells and decreased green fluorescent target cells (FIGs. 24E, 24F).

To see if the GD2-CAR T cells could kill target tumors for extended period, after the first coculture when all tumors cells were killed, more tumor cells were added to the GD2-CAR T cells to set up a second round killing, at E:T ratio = 1:3. Evident under fluorescent microscope, the results showed that GD2 CAR-T cells, but not control T cells or non-specific CD19-CAR T cells, were able to killed HOSw cells even with the increased tumor ratio after 12 days in culture (FIG. 24G).

Specific induction of PD-L1 on OS cells and PD-1 on GD2-CAR T.

PD-L1 expression is a key immune evasion mechanism of tumor cells[22]. Flow cytometry histogram overlays revealed basal expression of PD-L1 on surface of both HOS and U2OS cells (FIGs. 25A,25B). Upon specific interaction with GD2 CAR T cells, up-regulation of PD-L1 in the co-cultured OS cells was observed as illustrated by increased MFI for PD-L1 signal, as compared with the cells co-cultured with control T cells and CD19 CAR-T cells (FIGs. 25C,25D).

PD-L1 functions as a ligand of PD-1 related to immune checkpoint inhibition of T cells functions.

PD-1 expression was next examined on primary CAR T cells. The result showed that primary GD2 CAR T cells displayed increased PD-1 expression when compared to control T cells and non-specific CD19-CAR T cell after 1-day co-cultured with U2OSs and HOSw

cells (FIGs. 26A,26B,26C, and 26D,26E,26F, respectively, $p < 0.05$).

Example 4. Disialoganglioside (GD2)-specific chimeric antigen receptor T cells has a high potent anti-tumor activity on retinoblastoma cells

5 Retinoblastoma (Rb) is an aggressive eye cancer that is the most common malignant in infant and children. Decreased doses of chemotherapeutic treatments in infant are considered, however, Rb tumors trend to increase resistant to the drug. One of the alternative approaches is immunotherapy based on using chimeric antigen receptor (CAR)-engineered T cells targeting tumor-specific antigens that are highly expressed on Rb tumor, but are present
10 at low levels or not at all in normal tissues. Disialoganglioside 2 (GD2) protein might be a promising target antigen. Therefore, this study aims to investigate the killing efficiency of GD2-specific CAR T cells to eradicate Rb tumor cells *in vitro*.

MATERIALS AND METHODS

15 **Cell culture and reagents**

The human retinoblastoma cell line Y79 RB cells were cultured in suspension in RPMI-1640 medium (Gibco® Life Technologies, USA) supplemented with 15% (v/v) fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution, and maintained in a 37°C incubator with 5% CO₂. Cytokines recombinant human interleukin (IL)-2, IL-7, and IL-15 (
20 µg/ml) were purchased from a commercial vendor. AnnexinV-PE and propidium iodide (PI) were purchased from BD Biosciences (BD, USA). Carboplatin was purchased from Sigma.

Generation of GD2 specific chimeric antigen receptor lentiviral constructs

To engineer GD2-specific CARs, the humanized GD2-specific scFv clone, hu3F8 [15] was selected. The GD2 CAR sequence were then human codon-optimized and
25 chemically synthesized. To establish 4th generation CARs, several intracellular T cell signaling motifs were incorporated in the CAR including CD28 transmembrane and cytoplasmic domain, the co-stimulatory 4-1BB intracellular TRAF binding domain, the CD27 cytoplasmic domain, and the CD3ζ chain intracellular domain as illustrated in FIG. 12. The CAR gene was cloned into the lentiviral vector pTYF and packaged into lentiviral particles

for gene transfer.

hu3F8 scFv: (VH and VL linked by 218S linker which is underlined)

QVQLVESGPGVVQPGRSLRISCAVSGFSVTNYGVHWVRQPPGKGLEWLGVWAGGI
 5 TNYNSAFMSRLTISKDNSKNTVYLMNSLRAEDTAMYYCASRGGHYGYALDYWG
 QGTLVTVSSGSTSGSGKPGSSEGSTKGEIVMTQTPATLSVSAGERVTITCKASQSVSN
 DVTWYQQKPGQAPRLLIYSASNRYSGVPARFSGSGYGTEFTFTISSVQSEDFAVYFCQ
 QDYSSFGQGTKLEIK (SEQ ID NO: 19)

- 10 The above scFv domain was linked to the following CAR-inducible caspase 9 sequence
 (domain names are listed after each domain in bold);

FWVLVVVGGLACYSLLVTVAFIIFWV (**CD28 transmembrane domain**)
 KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL(**4-1BB intracellular
 domain**)

- 15 QRRKYRSNKGESPVPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP (**CD27
 intracellular domain**)

GSTSGSGKPGSSEGSTKG(**218S linker**)

RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDRRRGRDPGEMGGKPRRKNPQ
 EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP
 20 PR (**CD3zeta**)

GSTSGSGKPGSSEGSTKG (**218S linker**)

VGALESRLGNADLAYILSMEPCGHCLIINNPNFCRESGLRTRTGSNIDCEKLRRRFSSL
 HFMVEVKGDLTAKKMVLALLELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYG
 TDGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPEDSPGSN
 25 PEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI
 FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS (**truncated**

Casp9)

MGVQVETISPGDGRTPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFGFMLGKQE
 VIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE

(FKBP domain) (SEQ ID NO: 37)

Retinoblastoma cell line and T cells

Y79RB, a GD2 positive retinoblastoma cell line, and Jurkat T cell line were obtained
5 from American Type Culture Collection (ATCC). These cell lines were maintained in
RPMI1640 medium (Life Technologies, Inc. Grand Island, NY) supplemented with 10% fetal
bovine serum (Atlanta Biologicals, Inc. Norcross, GA), penicillin (100 units/ml) and
streptomycin (100ug/ml). Target cancer cell lines were transduced with lentiviral vectors
expressing a green fluorescent protein (wasabi GFP gene) and the reporter gene positive cells
10 were sorted by flow cytometry or selected with puromycin. Jurkat T cells were transduced
with lentiviral CAR vectors and the copy number of CAR per cell was determined by
quantitative PCR using genomic DNA.

Blood donors and primary T cell culture

Buffy coats from healthy donors (HDs) were purchased from LifeSouth Civitan Blood
15 Center (Gainesville, FL, USA) with approval of an Institutional Review Board. PBMCs were
isolated from buffy coats by gradient density centrifugation in Ficoll- Hypaque (GE
Healthcare Bio-Sciences AB, Piscataway, NJ, USA) as previously described [21]. T cells
were activated using anti-CD3 and anti-CD28 antibodies. The T cells were maintained in
TexMACS medium (Miltenyi Biotec Inc, San Diego, CA) supplemented with interleukin-2, -
20 7 and -15 as previously described²⁰. Phenotype analysis of the activated cells by flow
cytometry was performed to confirm T cell purity. After expansion for two to six days, the T
cells were transduced with lentiviral CAR vectors.

Lentiviral vector construction and CAR gene transduction

Lentiviral vectors were generated based on the NHP/TYF lentiviral vector system as
25 previously described [22,23]. CAR DNA was chemically synthesized and cloned into pTYF
transducing vector behind human EF1 α promoter. The final lentiviral-CAR constructs were
verified by restriction enzyme mapping and DNA sequencing.

CAR detection by PCR

The CAR transgene copy numbers in CART cells were determined by quantitative

SYBR green real time PCR (qRT-PCR) as previously described [20]. Genomic DNA was harvested from CART cells using Promega Wizard genomic DNA purification kit (Promega Corp. Madison, WI). The qRT-PCR reaction condition was as suggested by SABioscience and data collected using MX3000P (Stratagene, Agilent Technologies, Santa Clara, CA).

5 **Generation of GD2-specific chimeric antigen receptor (CAR) T-cells**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation, from healthy individuals. Lymphocytes were stimulated by phytohemagglutinin (PHA) treatment for 2-3 days and maintained in TexMACSTM medium (Miltenyi Biotec, CA, USA). Activated lymphocytes were transduced with CD19- or GD2-
10 specific lentiviral particles and maintained in TexMACSTM medium supplemented with human IL-2 (40 U/ml), IL-7 (20 U/ml), and IL-15 (10 U/ml) for 3-4 days.

Immunohistochemistry

Tumor samples from RB patients were fixed and embedded in paraffin blocks.

Tumor cell cytotoxicity assay

15 Y79 RB wasort cells (target (T) cells) were co-cultured with effector (E) T cells at various effector:target ratios (E:T) in the wells of 96-well flat-bottom plate at 37°C for 24 hours. Target cell lysis was monitored under a fluorescence microscope and the cells were collected. Cell death was analyzed using AnnexinV/PI staining by flow cytometry. Specific cell lysis was calculated as [(apoptosis of target cells in co-culture - spontaneous target cells
20 apoptosis)/ (100- spontaneous target cells apoptosis)] x 100. Spontaneous target cell death was considered as the percentage target cell death that cultured in T cell media without effector T cells. The results were representative of three independent experiments.

Flow cytometric analysis

To determine the expression of GD2 on the surface of primary neuroblastoma
25 (NB8858) cells and Y79 RB cells, the cells were stained with PE-conjugated mouse anti-human GD2 mAb clone 14.G2a (BD Biosciences, CA, USA). Antibody staining was monitored with a BD LSRII flow cytometer. Data analysis was carried out using FlowJo software (Tree Star Inc., Ashland, OR). For PD-L1 expression, cells were stained with anti-human CD274 or PD-L1 conjugated with PE-Cyanine7 monoclonal antibody clone MIHI

(eBiosciences, CA, USA).

AnnexinV/ PI staining

After co-culture experiment, cell culture samples were collected for analyzing tumor cells apoptosis by AnnexinV-PE/ PI staining. The cells were washed and stained with
5 AnnexinV-PE and PI (10 μ g/ml) according to manufacturer's instructions (BD Biosciences). The stained cells were analyzed by using flow cytometry on a LSRII. Every group was tested in triplicate.

Early and late apoptotic cells were defined in the population of AnnexinV+/PI-, and AnnexinV+/PI+ cells, respectively, while the necrotic cells were stained with PI+ only.

10 Percent of cell death includes early apoptosis, late apoptosis, and necrotic cells. Flow cytometry data was analyzed by using FlowJo software (Tree Star Inc., OR)

Surface staining and intracellular cytokine staining of CART cells

For effector functional analysis, the CART cells were mixed with target cells in an E/T ratio of 1:1 overnight with the addition of 1.5 μ l of FITC-conjugated anti-CD107a Ab. Positive
15 control used T cells (without CAR) stimulated with PMA (1 μ g/ μ l) and Ionomycin (1ng/ μ l) for 1 hour. The intracellular cytokines were immobilized after treated with monensin (6 μ g/ μ l) for 6 hours. The samples were then washed, blocked with 10% human and mouse sera for 30 min, stained with anti-CD4 and anti-CD8 Abs for 30 min, fixed and permeabilized with BD Fix/Perm Buffer, stained with anti-IFN- γ and anti-IL-2 Abs for 1 hour, and then analyzed by
20 flow cytometry. Data was collected on the BD LSRII flow cytometer and analyzed with Flowjo.

Effector cytokine analysis using cytokine bead array (CBA)

BD CBATM Human Soluble Protein Flex Set System was used to detect concentrations of cytokines IL-2, IL-6, TNF α and IFN- γ in the supernatants collected from
25 the CART cell killing assays on day 1 or 2 of incubation. The CBA system captures a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry. Each capture bead is coated with an antibody specific for a soluble protein. The detection reagent is a mixture of PE-conjugated antibodies, which provide a fluorescent signal in proportion to the amount of bound analyte. First, 10 tubes of

50μl set standard dilutions were prepared. Then 50μl of each unknown sample was added to an assay tube. 50μl of the mixed capture beads was added to each assay tube and incubated at room temperature for 1 hr. Then 50μl of the PE detection reagent was added and incubated for 2 hr at room temperature. 1 ml of wash buffer was added to each tube and centrifuged at 200g for 5 minutes. After the supernatant was removed, 300μl of wash buffer was added and vortexed prior to acquiring the results on flow cytometer.

Statistical analysis

All data are presented as mean $\pm$ SD. The significance of the difference between groups was evaluated by Unpaired Student t-test using Prism software (GraphPad, La Jolla, CA). A P value of less than 0.05 was considered statistically significant.

RESULTS

GD2 protein is highly expressed in retinoblastoma tumor samples and Y79 RB cell line

GD2 is a ganglioside protein that is highly expressed on the cell surface. Retinal tissue sample from eight Rb patients were examined for GD2 expression by using immunohistochemistry. Paraffin-embedded tissues were immunostained with anti-human GD2 antibody and detected by the standard peroxidase enzymatic method. FIG. 27A showed a strong positive signal of GD2 expression in all eight Rb samples and one of brain tumor, while there was no signal in normal brain tissue sample. The expression of GD2 on Y79 retinoblastoma cell line was also investigated by flow cytometry. The results demonstrated that almost 100% of Y79 RB cells expressed GD2 (FIG. 27B). Additionally, low expression of GD2 was found in primary neuroblastoma cells as previously reported. Expression of CD19 protein was not found on Y79 cells and thus the CD19 protein was used as a negative control along with CD19-specific CAR T cells for comparison with the GD2-specific CAR T cells.

Generation of primary T cells expressing GD2-specific CAR

A lentiviral vector was generated encoding a GD2-specific CAR (GD2-CAR), which consisted of (a) anti-GD2 scFv, (b) the hinge and transmembrane regions of the CD8 molecule, (c) the CD28, 4-1BB and CD27 costimulatory signaling moieties, and (d) the

cytoplasmic component of CD3 ζ molecule (FIG. 28A). Activated lymphocytes from a healthy donor were transduced with lentiviral particles encoding the GD2-CAR or the CD19-CAR, which were used to generate negative control T cells. To determine whether GD2-specific CARs were successfully transferred, the transduced cells were detected for CAR expression on cell surface of T-cells by using antibody. It was found that the CARs were expressed on the T-cells transduced with the lentiviral particles.

Tumor cell lysis by GD2-specific CAR T cells

To test whether GD2-specific CAR T cells could kill the tumor cells, Y79 Rb wasort (T) were co-cultured with CD19- or GD2-specific CAR T cells (E). Cell death was analyzed by using AnnexinV/PI staining as demonstrated in schematic diagram FIG. 28B. Co-culture experiment was performed at various E:T ratio to determine killing efficiency of CAR T cells. It was found that GD2-specific CAR T cells induced cell death (FIG. 29A, 29B) and specific lysis of the target cells (FIG. 29C). Killing of target Rb cells was observed both on day 1 of co-culture and day 3 of co-culture with the GD2-CAR T cells (FIG. 29D). Some killing was also observed at day 6 and day 14 of co-culture, although the degree of killing was less than on day 1 or day 3 (FIG. 30). Overall, the GD2-CAR T cells were able to effectively kill the Rb cells.

It was determined that GD2 was downregulated in tumor cells upon GD2-CAR T cell co-culture (FIG. 31A) and that GD2-resistant Rb cells developed (FIG. 31B). It was also shown that PD-L1 expression increased upon GD2-CAR T cell co-culture (FIG. 31C). This may indicate that treatment with PD-L1 inhibitors or PD1 inhibitors may increase efficacy of the GD2-CAR T cells. Overall, however, the GD2-CAR T cells were able to effectively kill the Rb cells, indicating that such T cells are useful for treating Rb, either alone or potentially in combination with a PD-L1 or PD1 inhibitor.

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated

otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications of the disclosure to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, i.e., “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); *etc.*

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or

“exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element
5 selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or
10 unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and
15 optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); *etc.*

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts
20 of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, i.e., to mean including
25 but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03. It should be appreciated that embodiments described in this document using an open-ended transitional phrase (*e.g.*, “comprising”) are also contemplated, in alternative embodiments, as “consisting

of” and “consisting essentially of” the feature described by the open-ended transitional phrase. For example, if the disclosure describes “a composition comprising A and B”, the disclosure also contemplates the alternative embodiments “a composition consisting of A and B” and “a composition consisting essentially of A and B”.

Claims

What is claimed is:

1. A chimeric antigen receptor (CAR) comprising:
5 an antigen binding domain;
a transmembrane domain; and
a cytoplasmic domain containing an interleukin 15-receptor α (IL-15R α) cytoplasmic domain.
- 10 2. The CAR of claim 1, wherein the transmembrane domain is a CD28 or CD8 transmembrane domain.
3. The CAR of claim 1 or claim 2, wherein the cytoplasmic domain further comprises a CD3zeta signal transduction domain.
- 15 4. The CAR of any one of claims 1 to 3, wherein the cytoplasmic domain further comprises a CD27 signaling domain.
5. The CAR of any one of claims 1 to 4, wherein the antigen binding domain is a single-
20 chain variable fragment (scFv).
6. The CAR of claim 5, wherein the CAR comprises a CD28 transmembrane domain, an IL-15R α cytoplasmic domain, and a CD3zeta signal transduction domain.
- 25 7. The CAR of claim 6, wherein the CAR further comprises a first spacer between the CD28 transmembrane domain and the IL-15R α cytoplasmic domain and a second spacer between the IL-15R α cytoplasmic domain and the CD3zeta signal transduction domain.
8. The CAR of any one of claims 1 to 7, wherein the antigen binding domain is specific

for CD19.

9. A nucleic acid comprising a sequence that encodes the CAR of any one of claims 1 to 8.

5

10. An immune cell comprising a CAR of any one of claims 1 to 8 and/or the nucleic acid of claim 9.

11. The immune cell of claim 10, wherein the immune cell is a T cell or NK cell.

10

12. The immune cell of claim 11, wherein the immune cell is a T cell.

13. A composition comprising a plurality of the immune cell of any one of claims 10 to 12.

15

14. The composition of claim 13, further comprising a pharmaceutically acceptable carrier.

15. A method of generating a plurality of CAR-modified immune cells, the method comprising:

20

introducing a lentiviral vector comprising the nucleic acid of claim 9 into a plurality of immune cells.

16. The method of claim 15, wherein the immune cells are T cells.

25

17. A method of treating a subject having a disease, the method comprising:

administering the immune cell of any one of claims 10 to 12, the composition of claim 13 or 14, or the plurality of immune cells produced by the method of claim 15 or 16 into a subject having a disease or at risk of having a disease.

18. The method of claim 17, wherein the disease is cancer, an autoimmune disease or an infection.

5 19. The method of claim 18, wherein the disease is cancer.

20. A chimeric antigen receptor (CAR) comprising:
an antigen binding domain;
a transmembrane domain; and
10 a cytoplasmic domain containing a CD27 intracellular domain.

21. The CAR of claim 20, wherein the transmembrane domain is a CD28 or CD8 transmembrane domain.

15 22. The CAR of claim 20 or claim 21, wherein the cytoplasmic domain further comprises a 4-1BB intracellular domain.

23. The CAR of any one of claims 20 to 22, wherein the cytoplasmic domain further comprises a CD3zeta signal transduction domain.

20 24. The CAR of any one of claims 20 to 23, wherein the cytoplasmic domain further comprises an iCasp9 domain and/or a FKBP domain.

25 25. The CAR of any one of claims 20 to 24, wherein the antigen binding domain is a single-chain variable fragment (scFv).

26. The CAR of claim 24, wherein the CAR comprises a CD28 transmembrane domain, cytoplasmic domain comprising a CD27 intracellular domain and a 4-1BB intracellular domain, and a CD3zeta signal transduction domain.

27. The CAR of claim 24, further comprising an iCasp9 domain and/or a FKBP domain.

28. The CAR of claim 27, wherein the CAR further comprises a first spacer between the
5 CD28 transmembrane domain and the cytoplasmic domain comprising a CD27 intracellular domain and a 4-1BB intracellular domain, and a second spacer between the cytoplasmic domain comprising a CD27 intracellular domain and a 4-1BB intracellular domain and the CD3zeta signal transduction domain.

10 29. The CAR of claim 28, further comprising a third spacer between the CD3zeta signal transduction domain and the iCasp9 domain and/or a FKBP domain.

30. The CAR of any one of claims 20 to 29, wherein the antigen binding domain is specific for CD30.

15 31. A nucleic acid comprising a sequence that encodes the CAR of any one of claims 20 to 30.

32. An immune cell comprising a CAR of any one of claims 20 to 30 and/or the nucleic
20 acid of claim 31.

33. The immune cell of claim 32, wherein the immune cell is a T cell or NK cell.

34. The immune cell of claim 33, wherein the immune cell is a T cell.

25 35. A composition comprising a plurality of the immune cell of any one of claims 32 to 34.

36. The composition of claim 35, further comprising a pharmaceutically acceptable

carrier.

37. A method of generating a plurality of CAR-modified immune cells, the method comprising:

5 introducing a lentiviral vector comprising the nucleic acid of claim 26 into a plurality of immune cells.

38. The method of claim 37, wherein the immune cells are T cells.

10 39. A method of treating a subject having a disease, the method comprising:

administering the immune cell of any one of claims 32 to 34, the composition of claim 35 or 36, or the plurality of immune cells produced by the method of claim 37 or 38 into a subject having a disease or at risk of having a disease.

15 40. The method of claim 39, wherein the disease is cancer, an autoimmune disease or an infection.

41. The method of claim 40, wherein the disease is cancer.

20 42. The method of any one of claims 39 to 41, wherein the disease is a CD30⁺ cancer.

43. A chimeric antigen receptor (CAR) comprising:

an antigen binding domain specific for GD2;

a transmembrane domain; and

25 a cytoplasmic domain containing one or more of a CD27 signaling domain, a 4-1BB intracellular domain, and a CD3zeta signal transduction domain.

44. The CAR of claim 43, wherein the transmembrane domain is a CD28 or CD8 transmembrane domain.

45. The CAR of claim 43 or claim 44, wherein the cytoplasmic domain contains two or more of a CD27 signaling domain, a 4-1BB intracellular domain, and a CD3zeta signal transduction domain.

5

46. The CAR of any one of claims 43 to 45, wherein the cytoplasmic domain contains a CD27 signaling domain, a 4-1BB intracellular domain, and a CD3zeta signal transduction domain.

10

47. The CAR of any one of claims 43 to 46, wherein the antigen binding domain is a single-chain variable fragment (scFv) or an antigen binding antibody domain.

48. The CAR of any one of claims 43 to 46, wherein the antigen binding domain is a single-chain variable fragment (scFv).

15

49. A nucleic acid comprising a sequence that encodes the CAR of any one of claims 43 to 48.

20

50. An immune cell comprising a CAR of any one of claims 43 to 48 and/or the nucleic acid of claim 49.

51. The immune cell of claim 50, wherein the immune cell is a T cell or NK cell.

52. The immune cell of claim 51, wherein the immune cell is a T cell.

25

53. A composition comprising a plurality of the immune cell of any one of claims 50 to 52.

54. The composition of claim 52, further comprising a pharmaceutically acceptable

carrier.

55. A method of generating a plurality of CAR-modified immune cells, the method comprising:

5 introducing a lentiviral vector comprising the nucleic acid of claim 49 into a plurality of immune cells.

56. The method of claim 55, wherein the immune cells are T cells.

10 57. A method of treating a subject having cancer, the method comprising:
administering the immune cell of any one of claims 50 to 52, the composition of claim 53 or 54, or the plurality of immune cells produced by the method of claim 55 or 56 to a subject having cancer.

15 58. The method of claim 55, wherein the cancer is osteosarcoma or retinoblastoma.

59. The method of claim 57 or 58, wherein the method further comprises administering a PD-L1 or PD1 inhibitor to the subject.

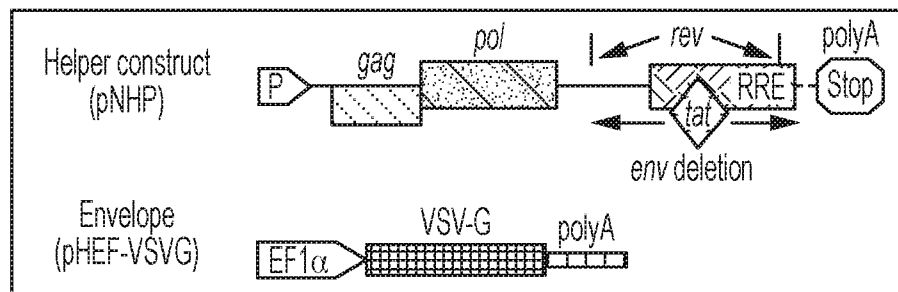
20 60. A method of treating a subject having cancer, the method comprising:
administering (a) an immune cell expressing a CAR that targets GD2 and (b) a PD-L1 or PD1 inhibitor to a subject having cancer.

61. The method of claim 60, wherein the cancer is osteosarcoma or retinoblastoma.

25 62. The method of claim 60 or 61, wherein the immune cell is a T cell.

63. The method of any one of claims 60 to 62, wherein the immune cell is a plurality of immune cells in a composition.

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Transducing vectors (pTYF-CAR)

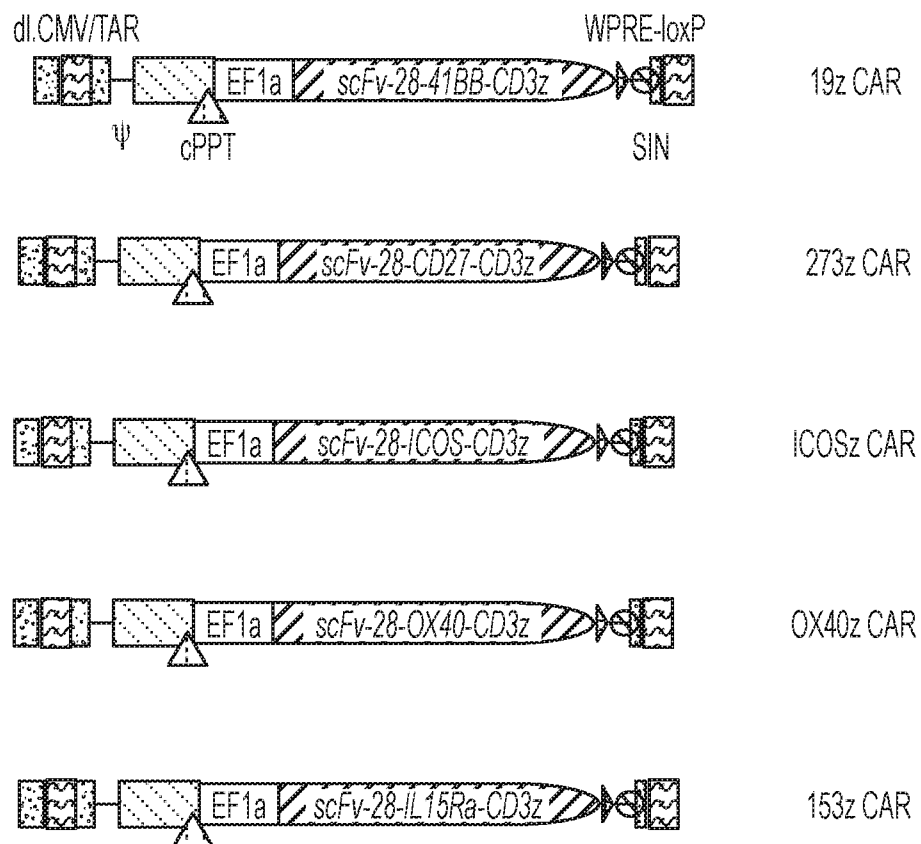


FIG. 1

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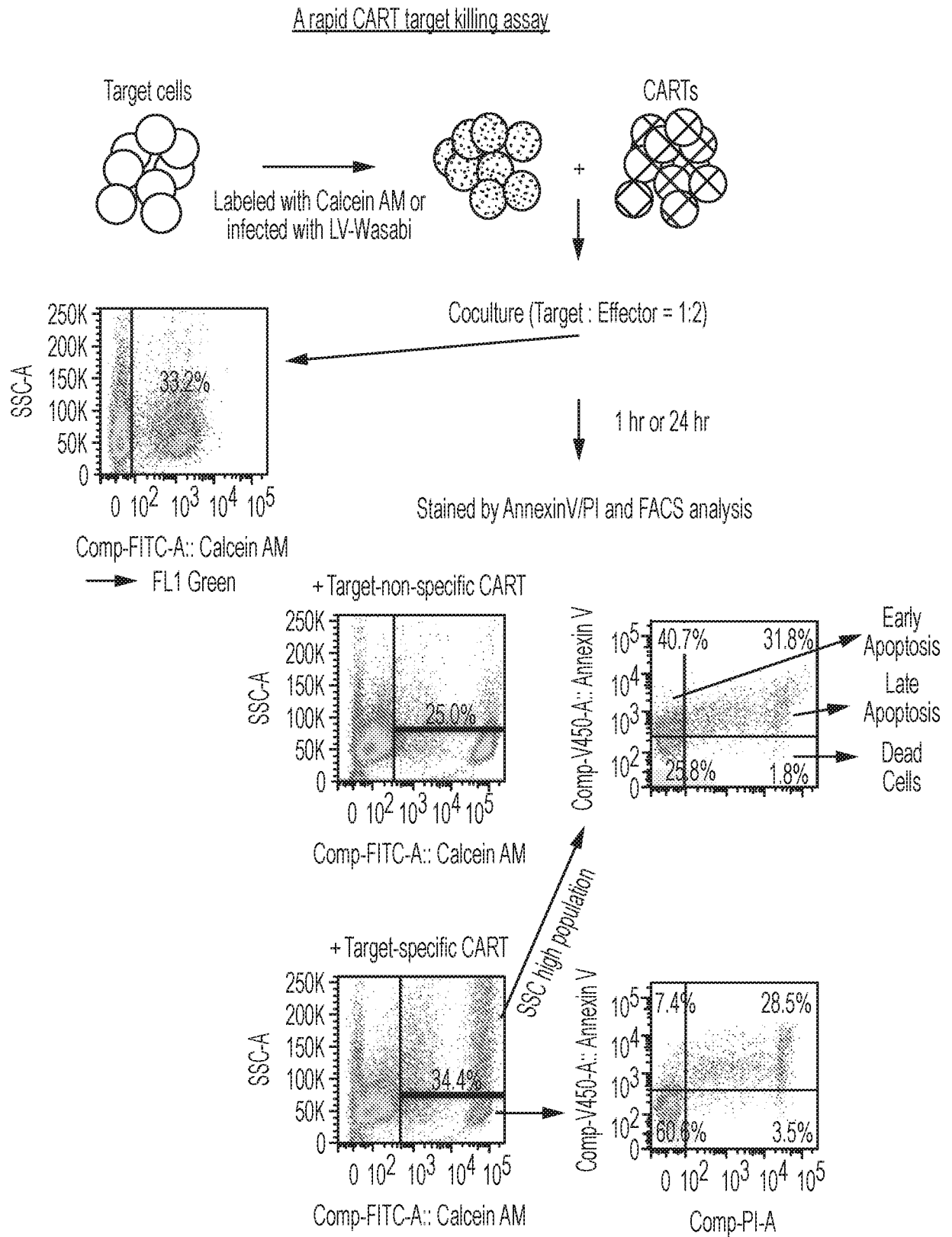
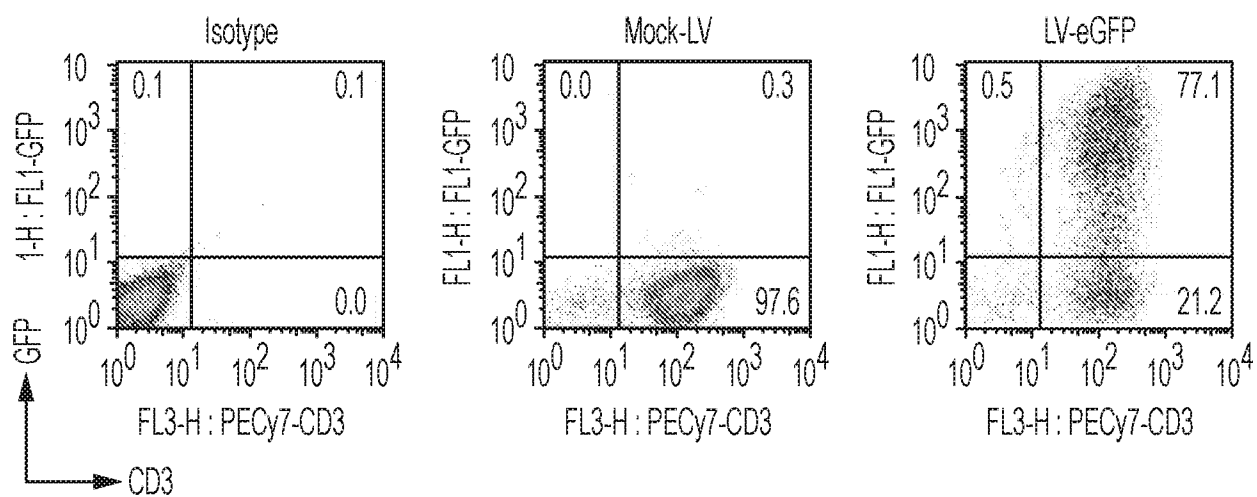
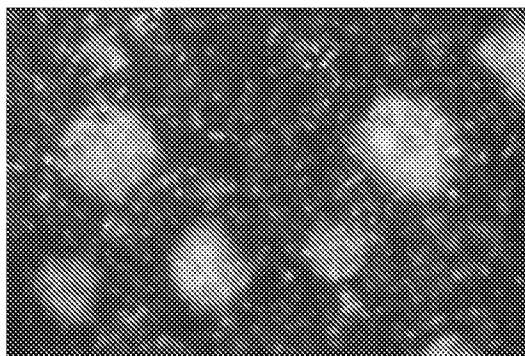


FIG. 2

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Efficient LV gene transfer into human T cells

Day 6 after LV-eGFP



Day 13 after LV-eGFP

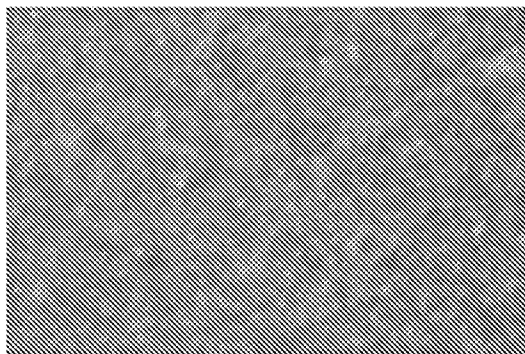


FIG. 3

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IC-CAR T cells + RS4;11-GFP B-ALL cells (1 day)

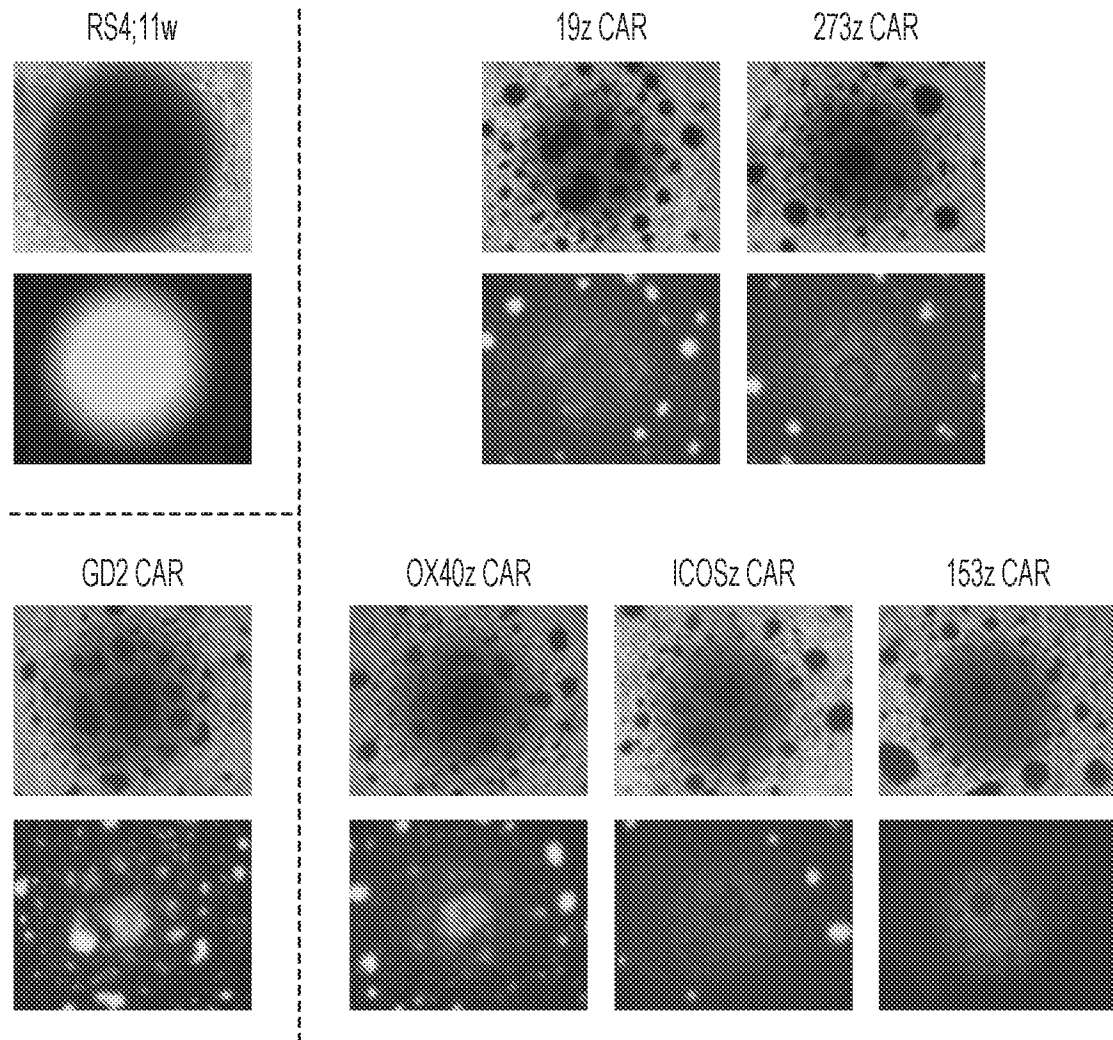


FIG. 4

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CARTs of healthy donor target killing assay

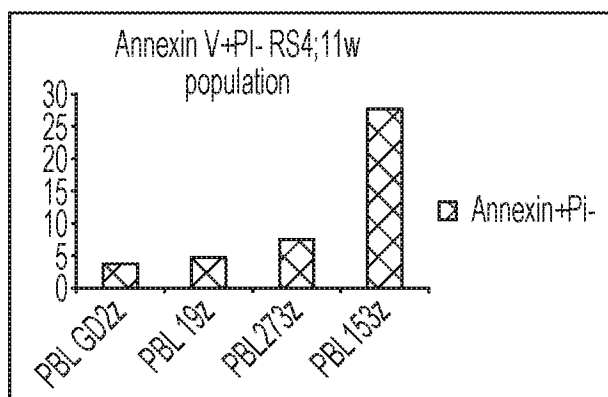
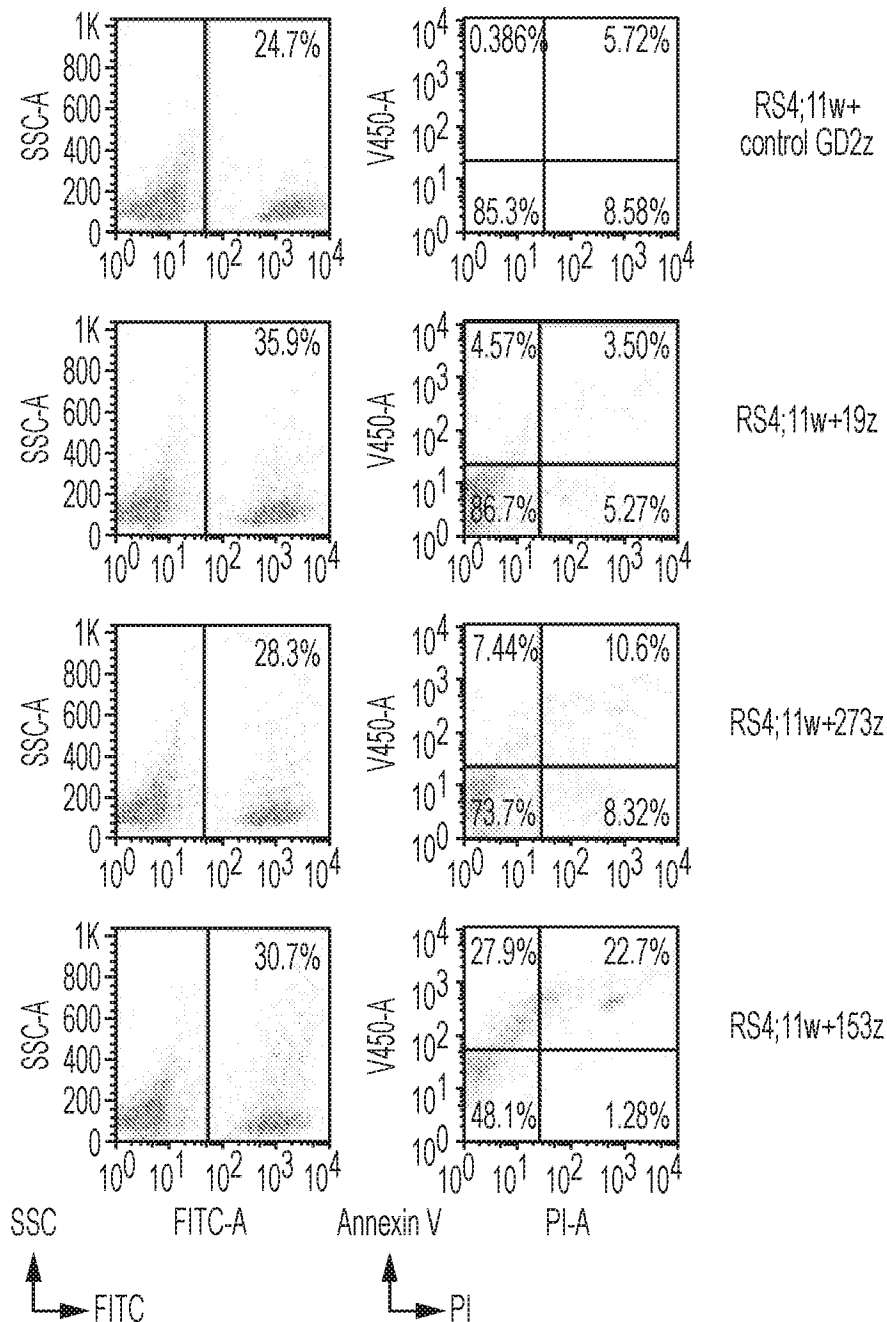


FIG. 5A

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CARTs of healthy donor target killing assay

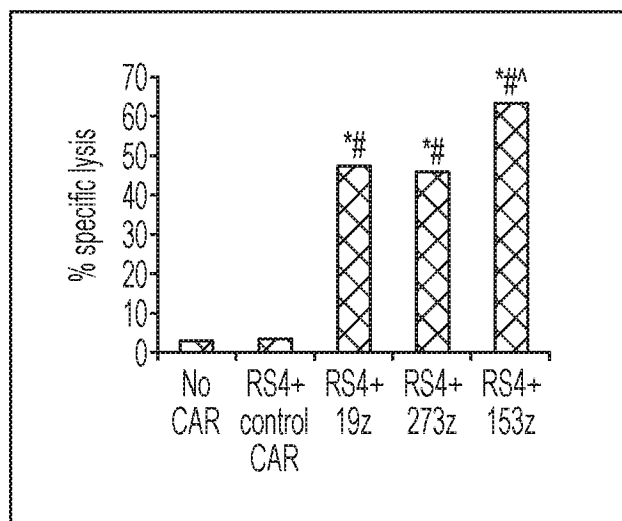
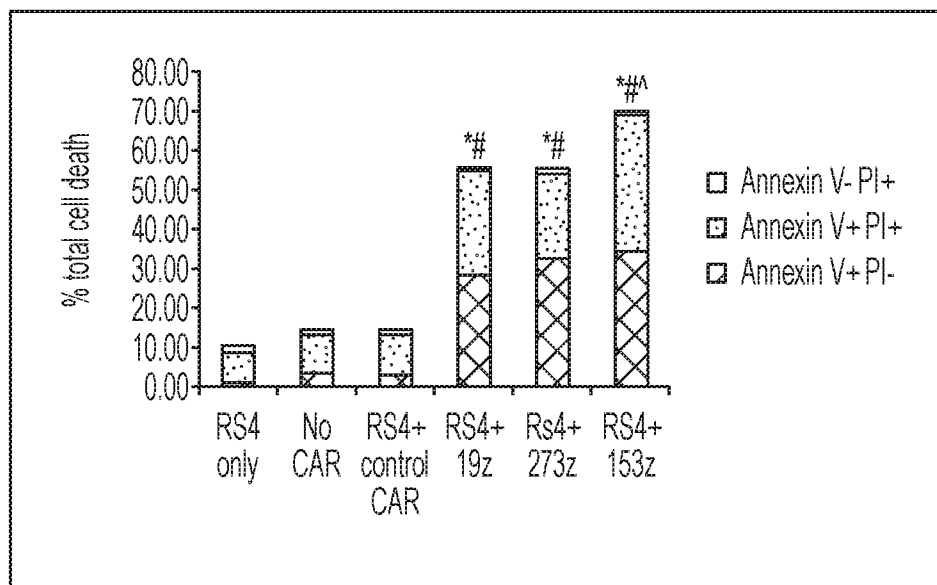


FIG. 5B

CARTs of leukemia patients target killing assay

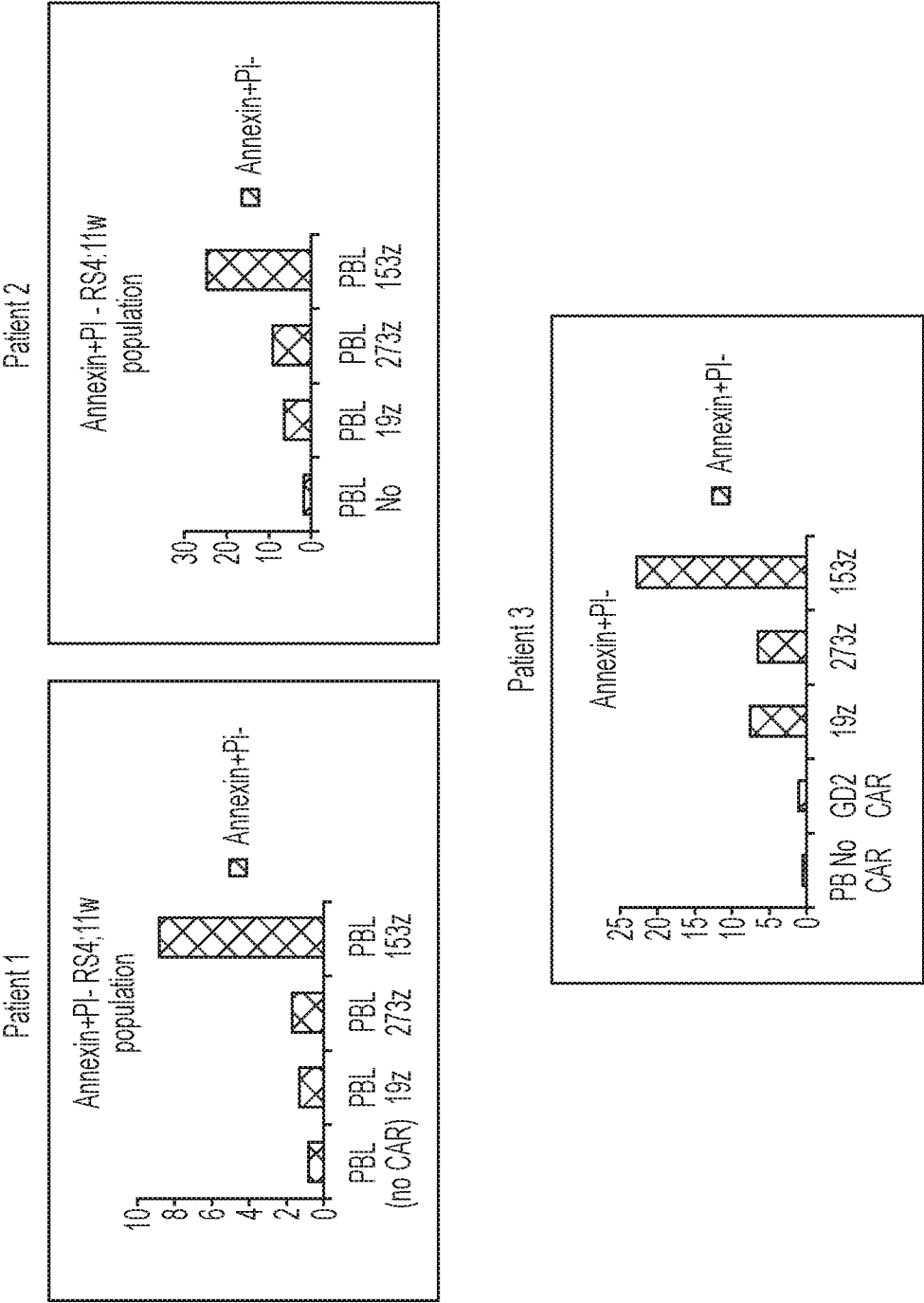


FIG. 6A

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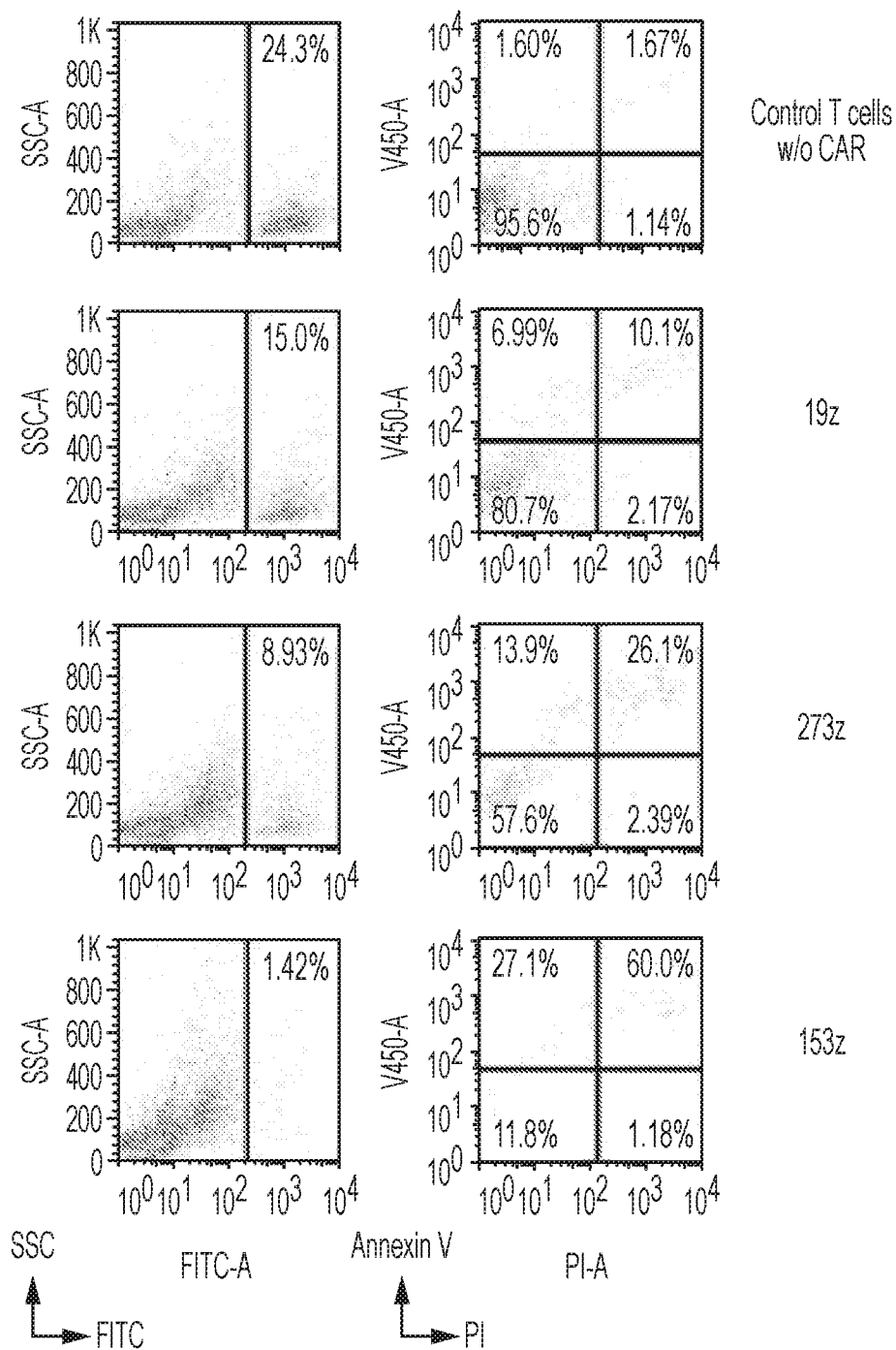


FIG. 6B

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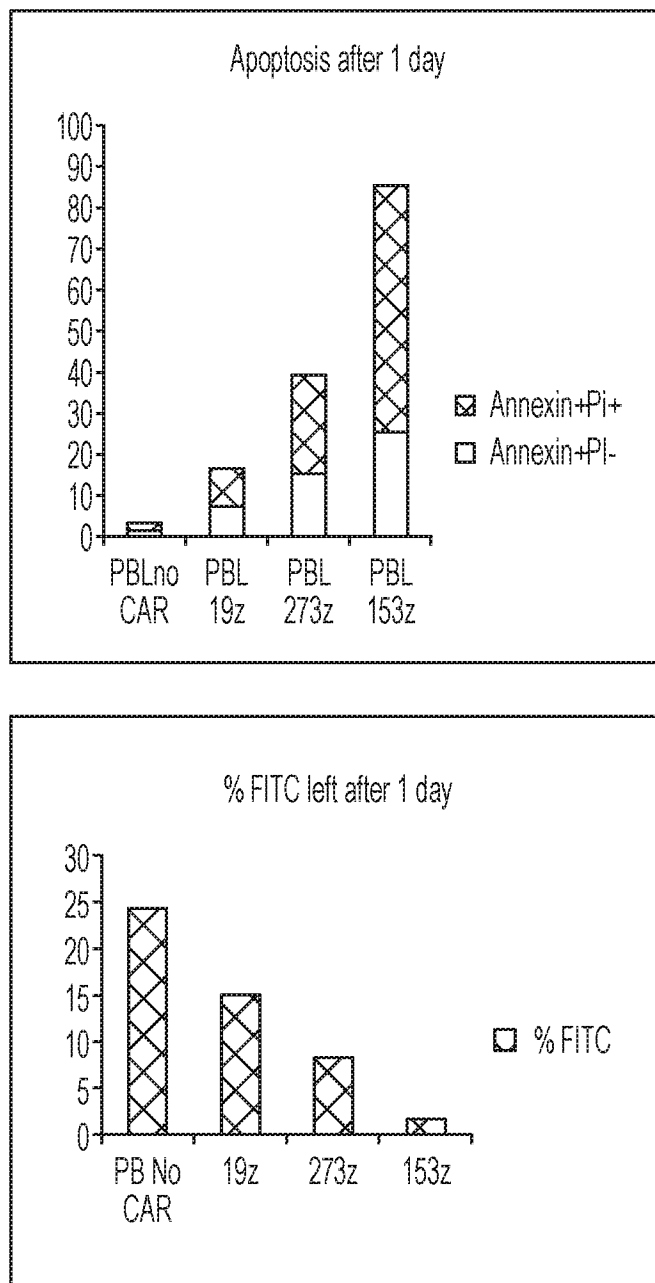


FIG. 6B
(CONTINUED)

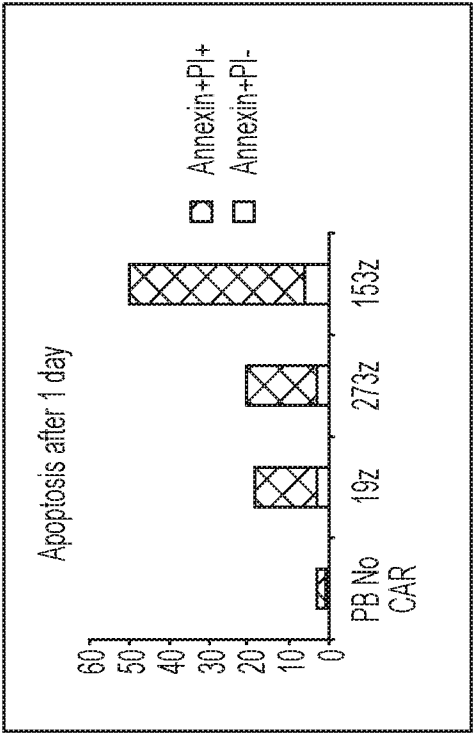
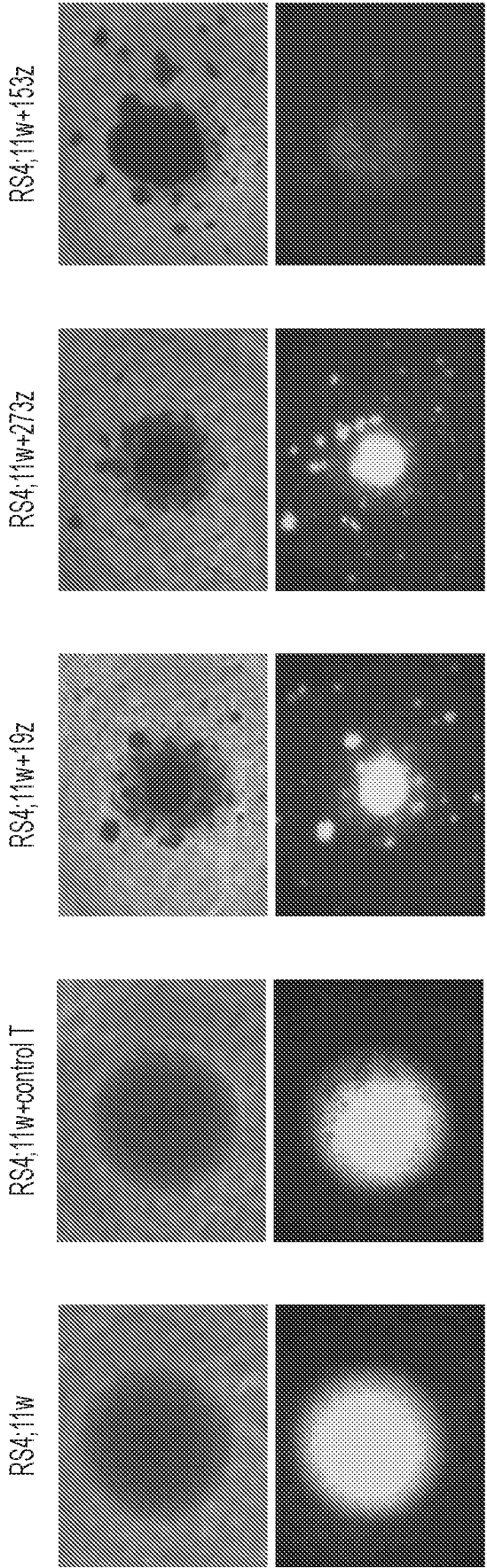
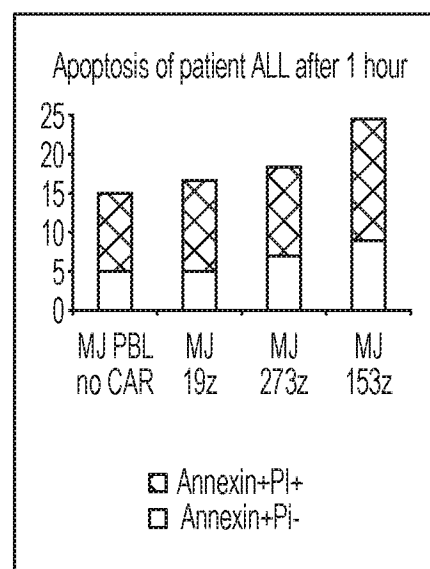
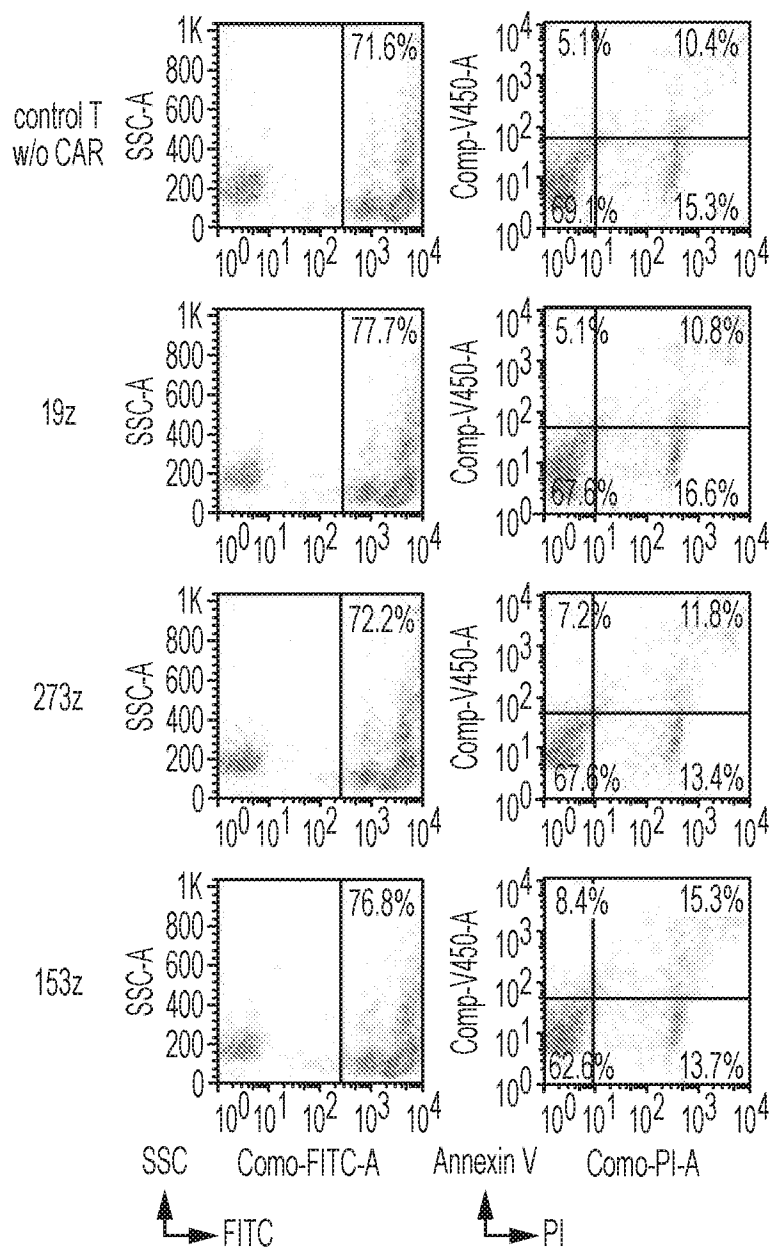


FIG. 6C

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Autologous patient ALL CART killing assay



Calcein AM labeled ALL

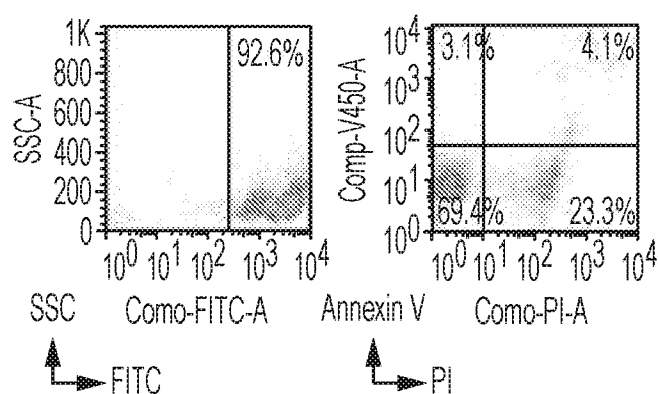
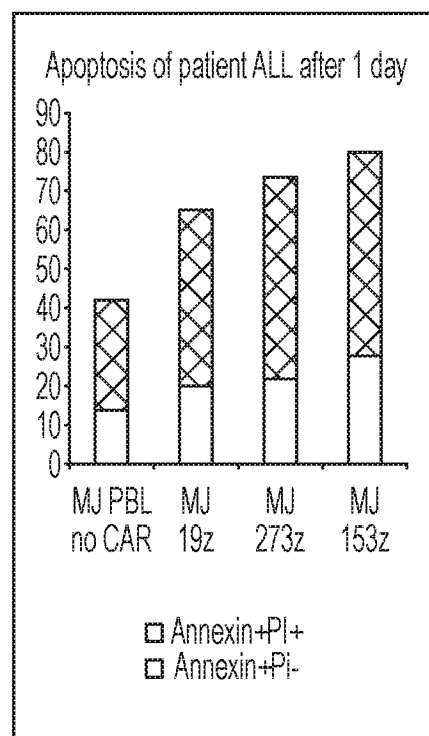
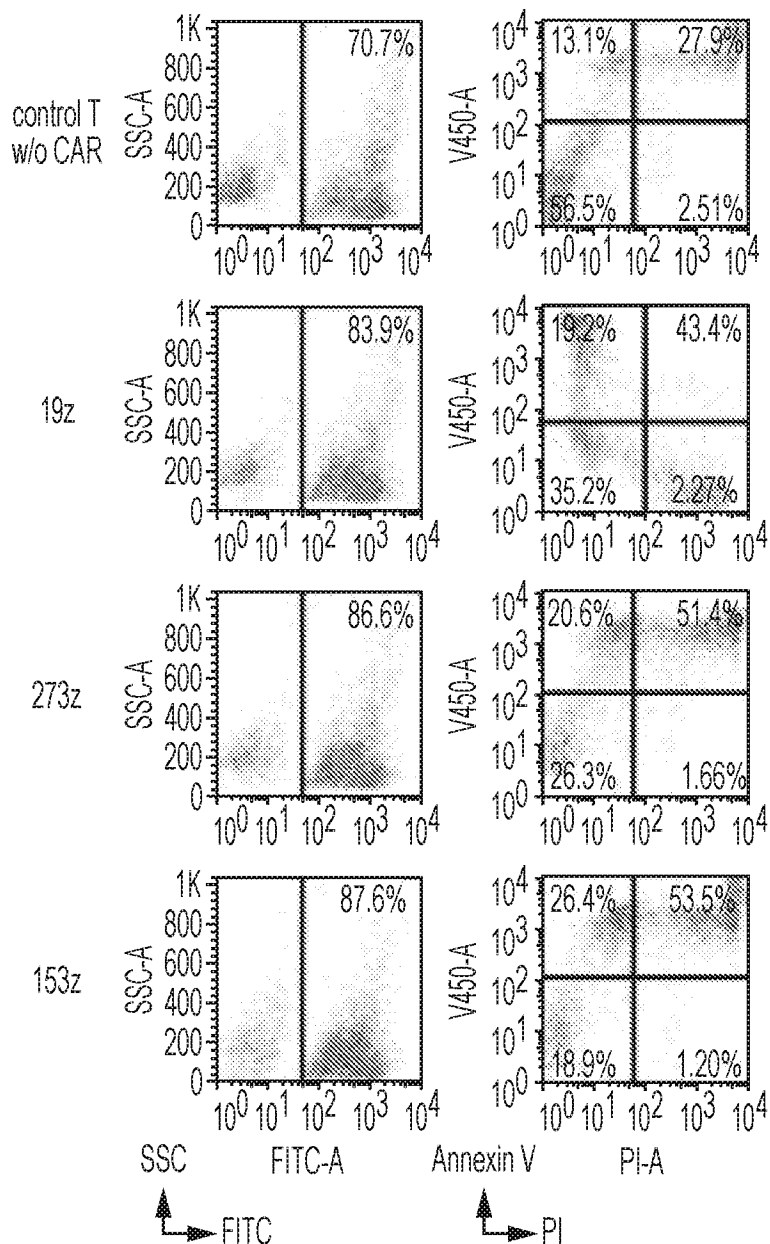


FIG. 7A

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Autologous patient ALL CART killing assay



Calcein AM labeled ALL

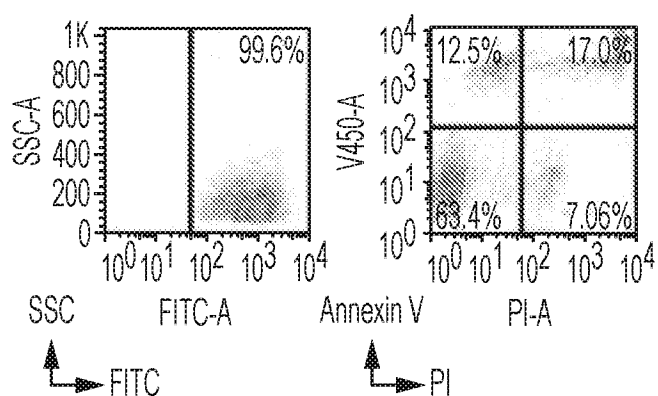


FIG. 7B

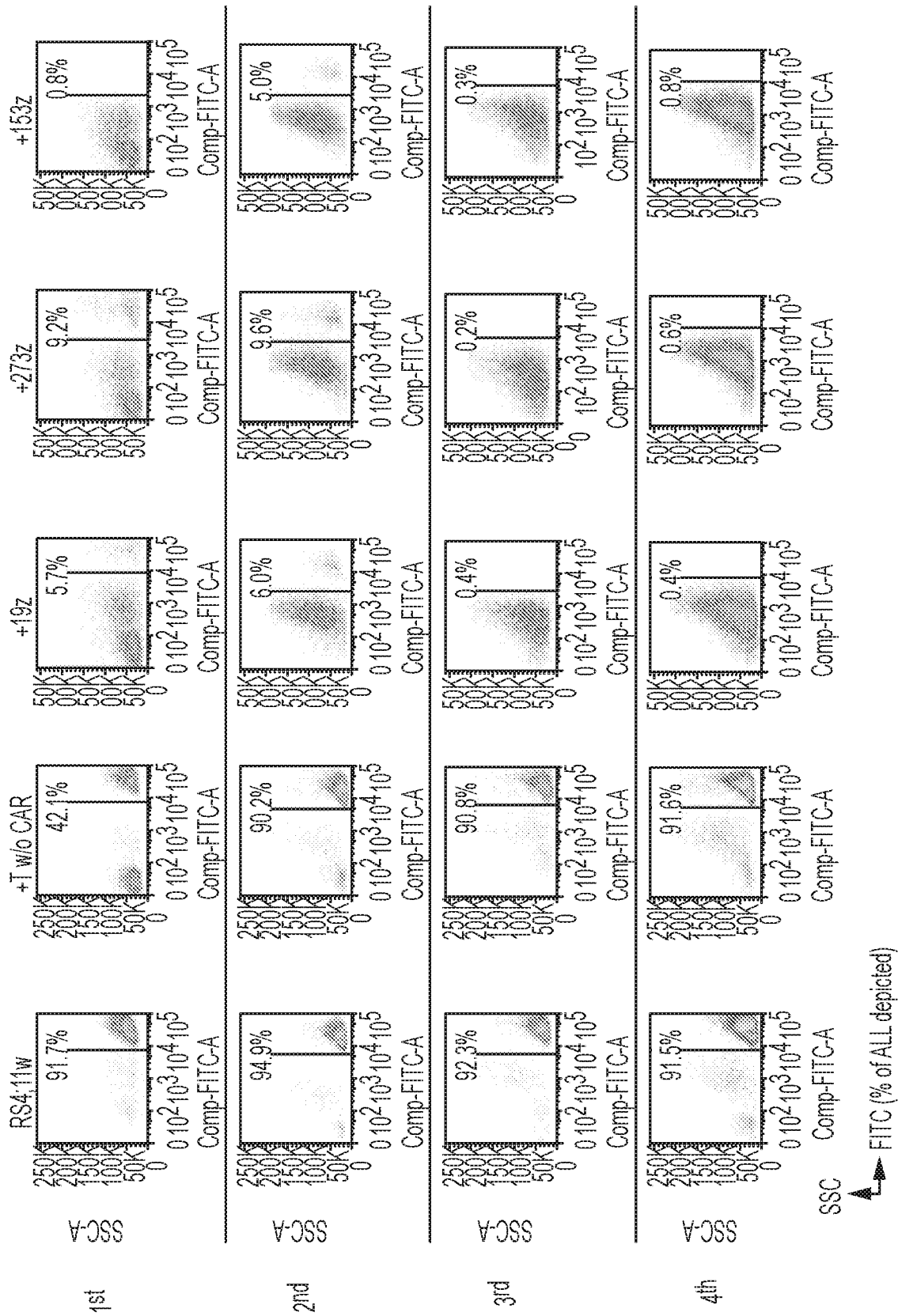


FIG. 8

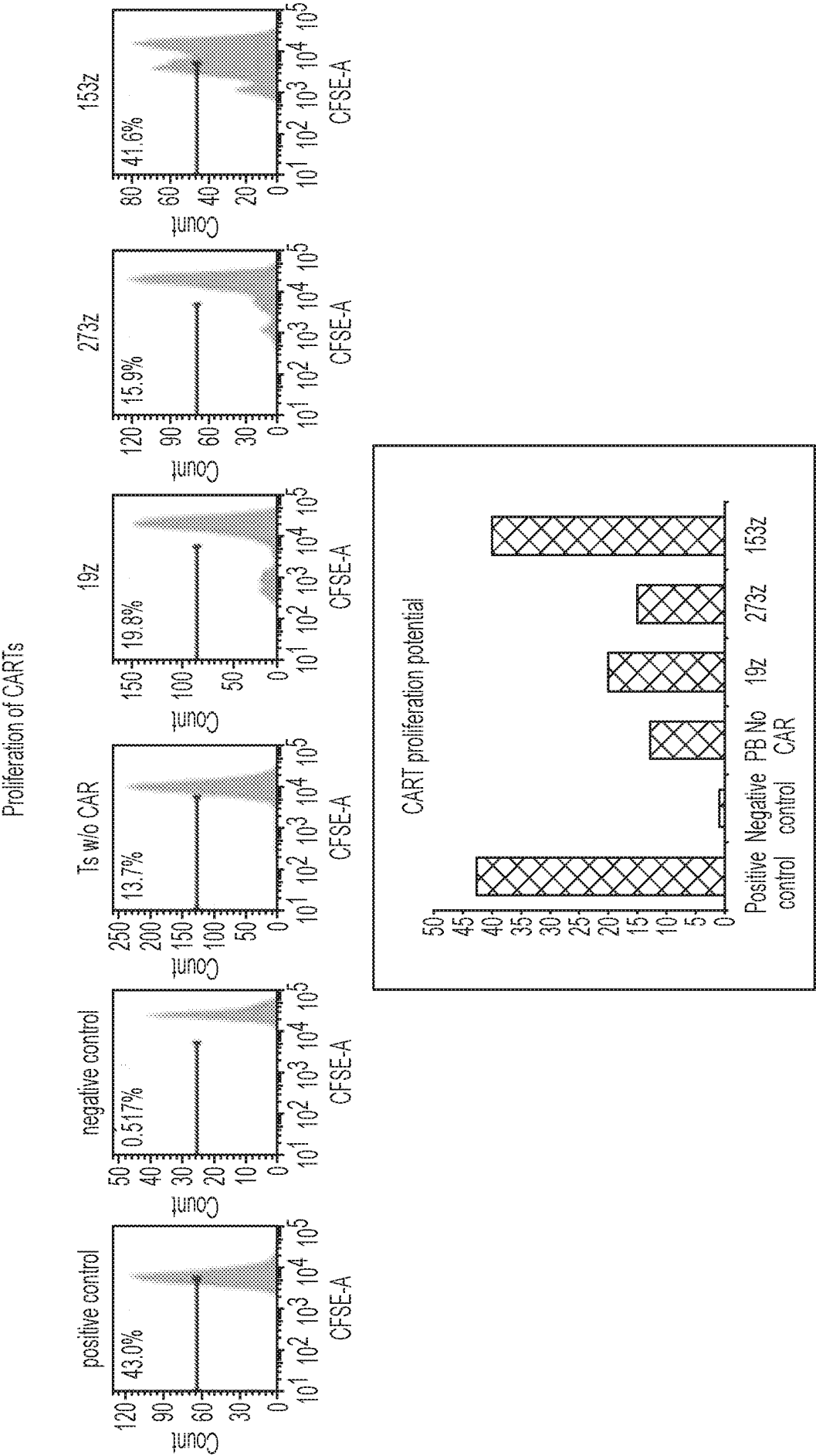


FIG. 9

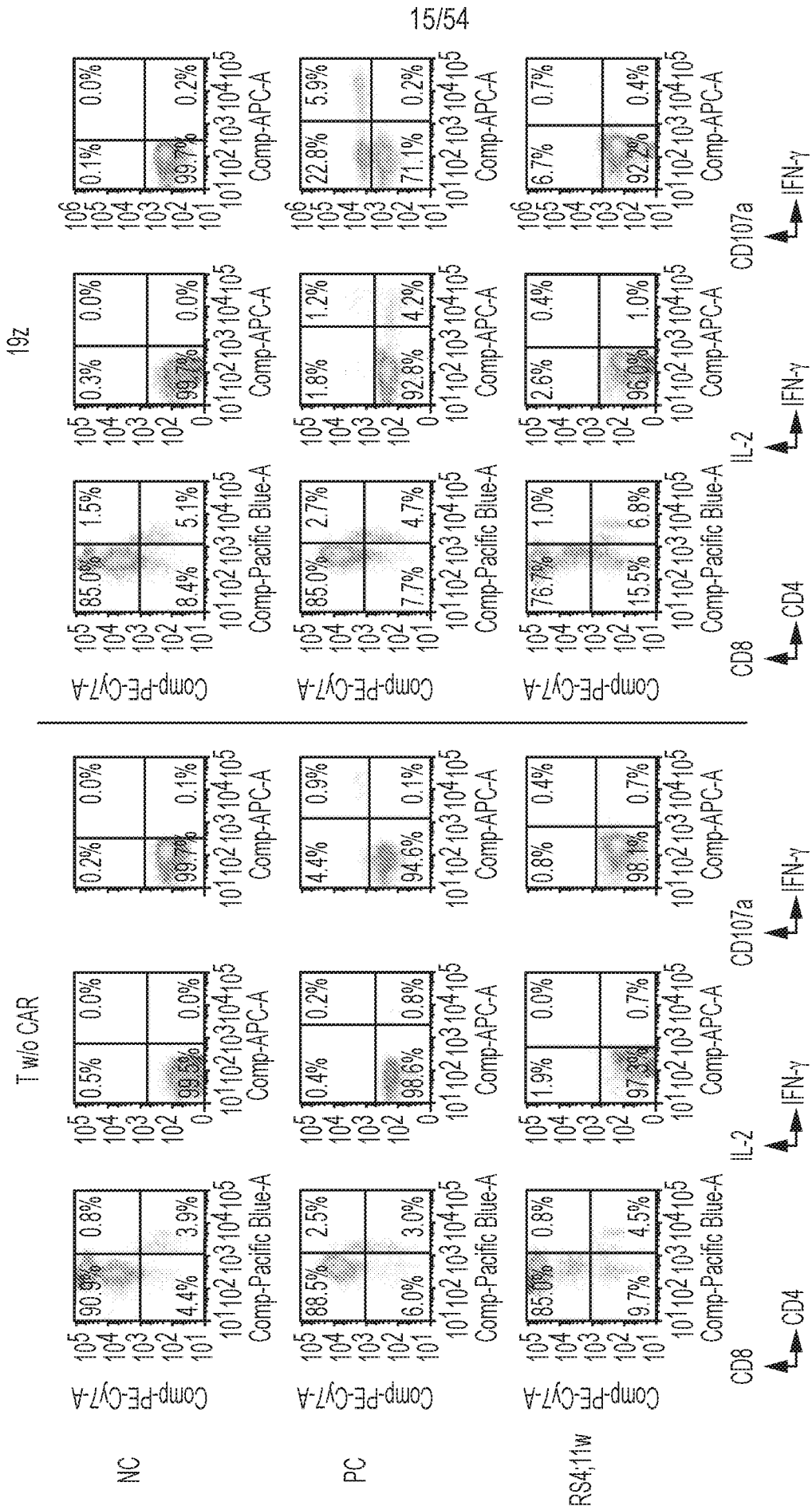


FIG. 10A

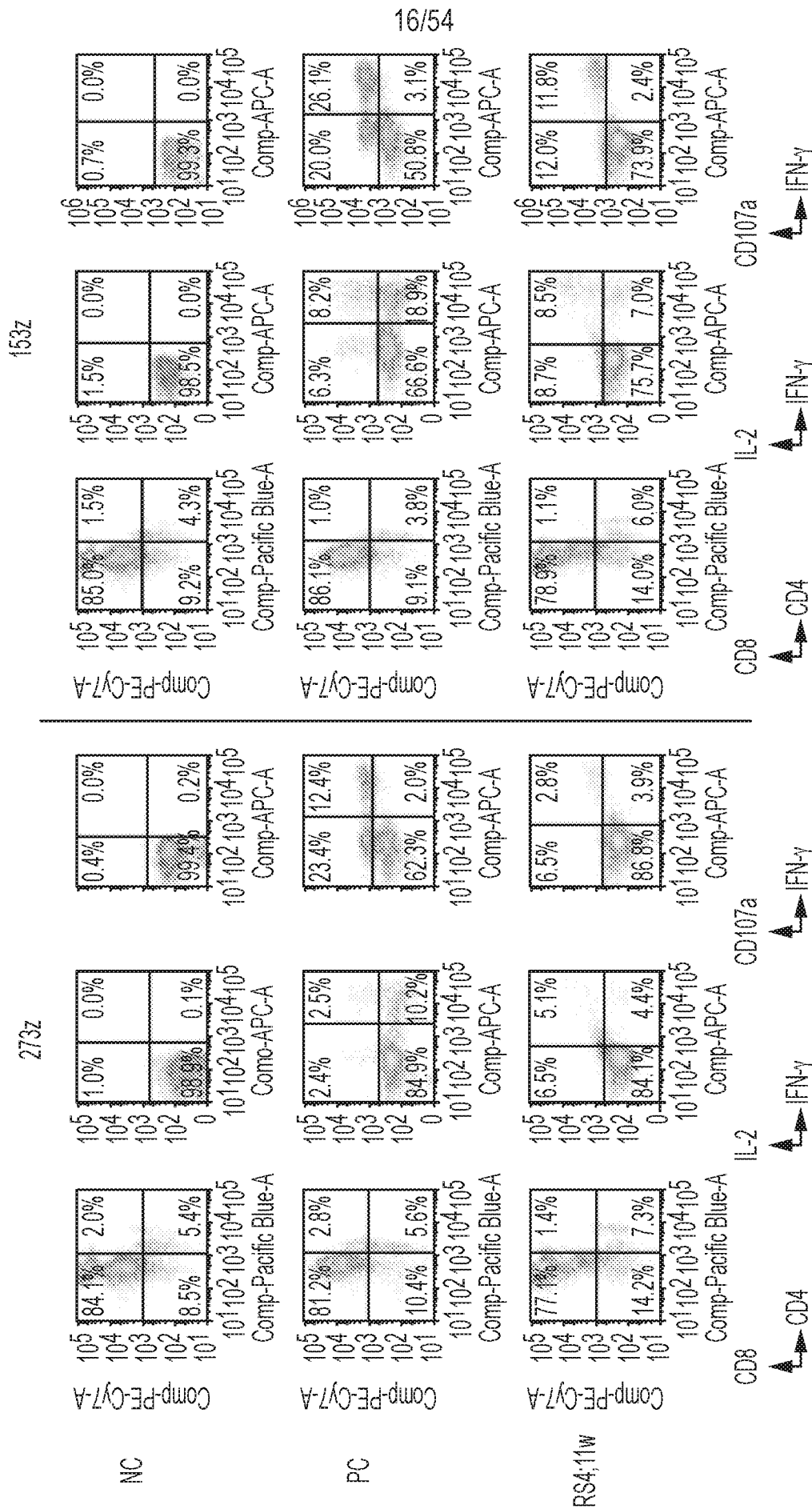


FIG. 10B

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Tumor-stimulated cytokine expression

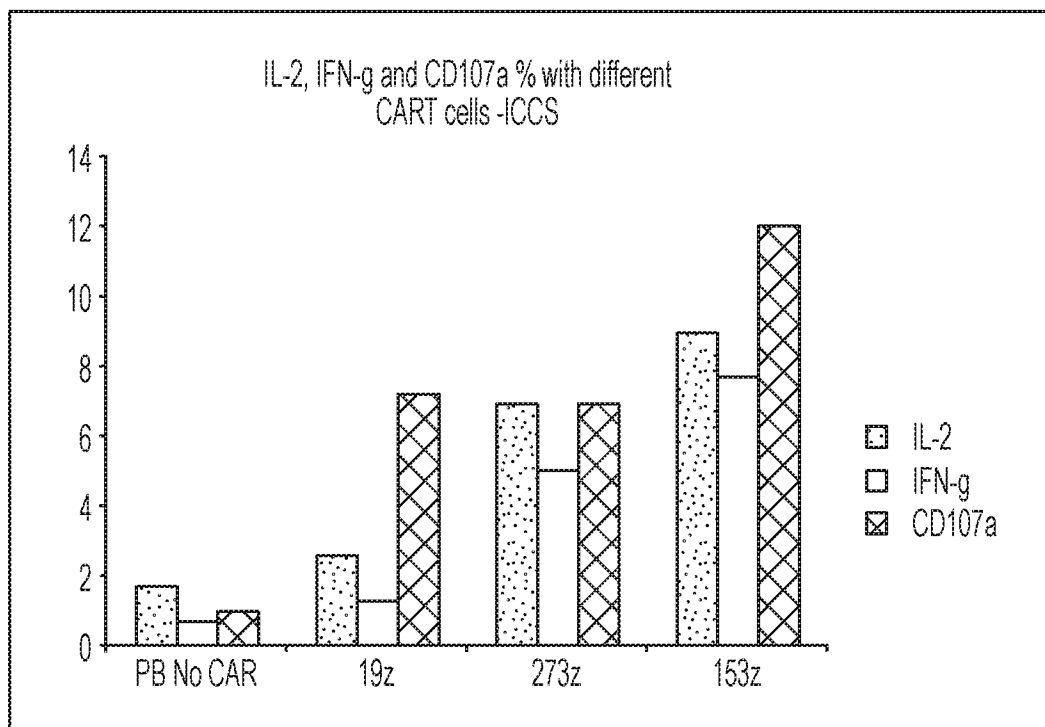


FIG. 10C

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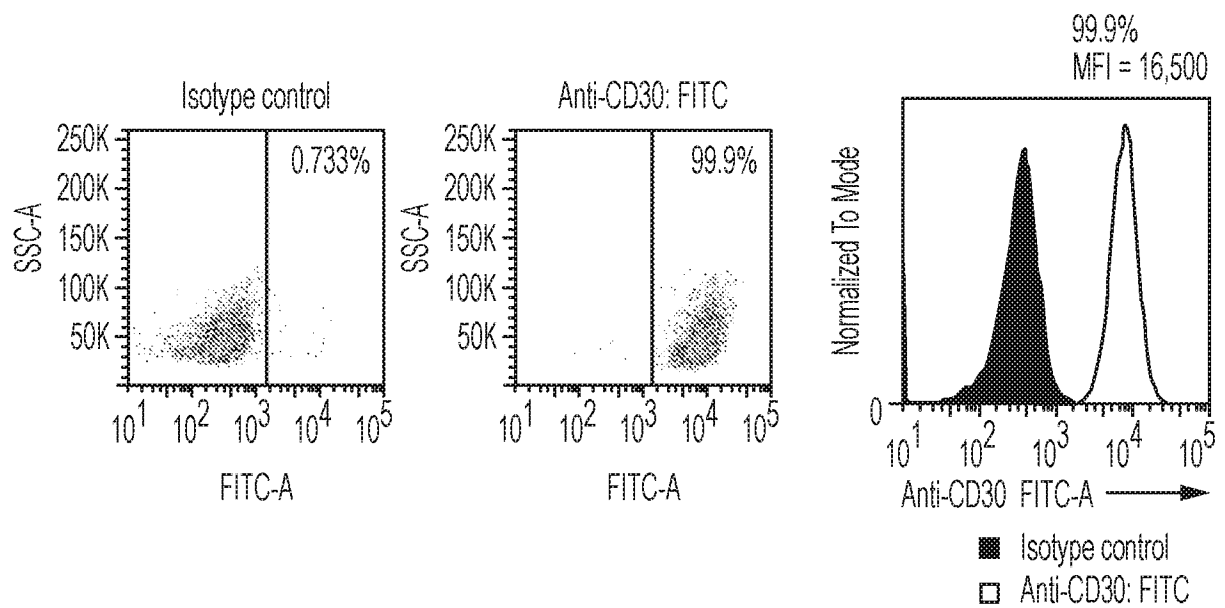
Flow cytometry analysis of CD30+ primary tumor cells

FIG. 11

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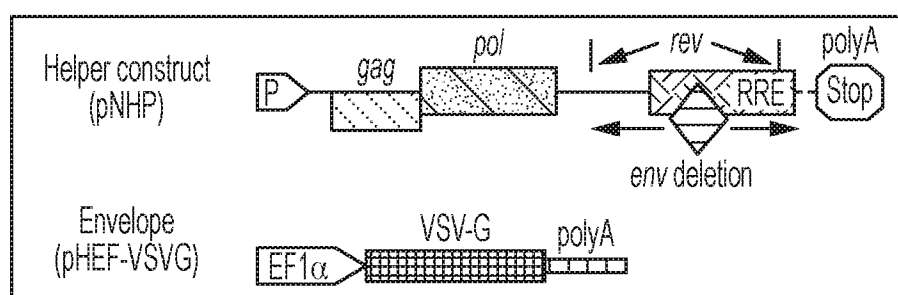


FIG. 12

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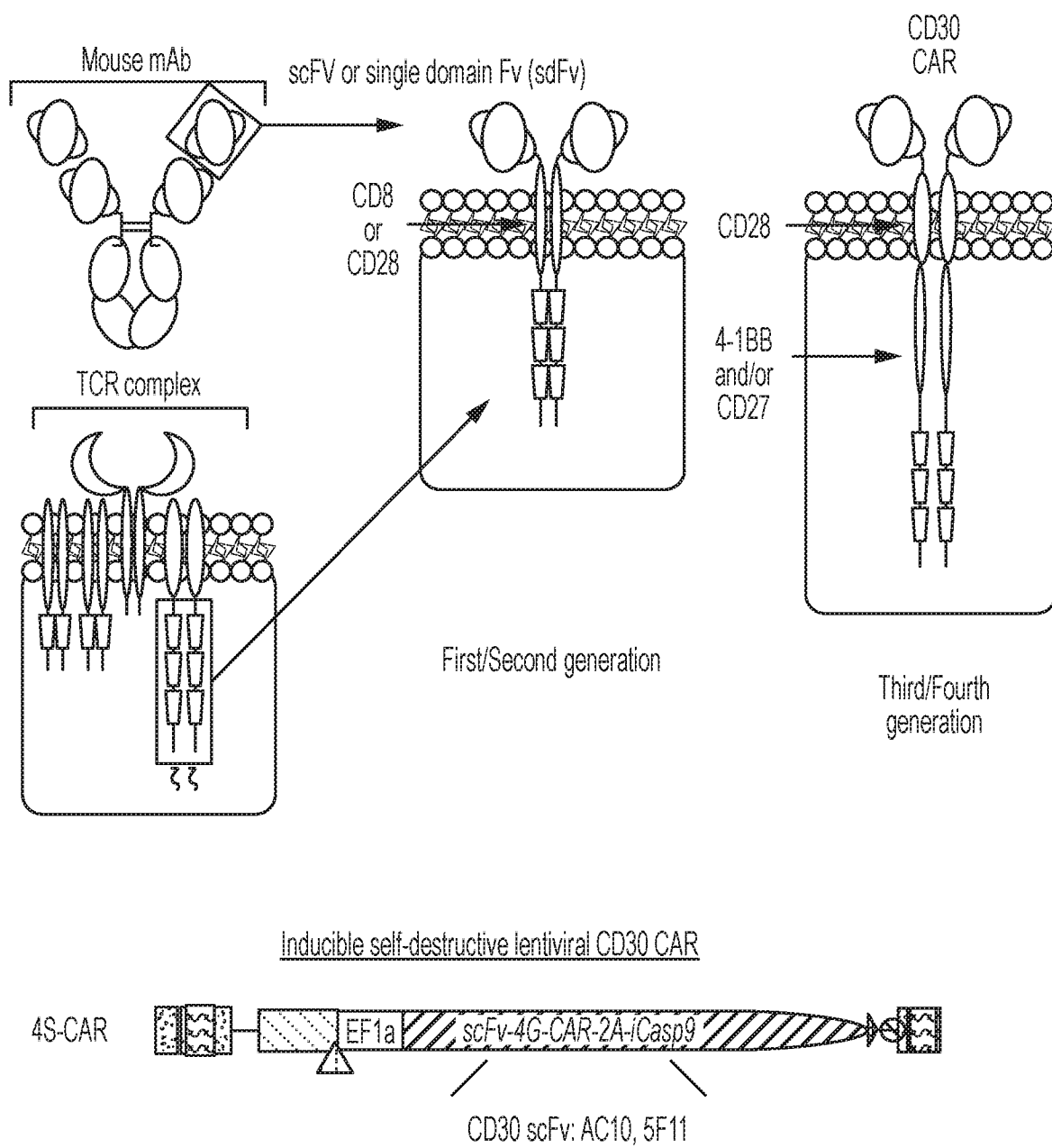


FIG. 13

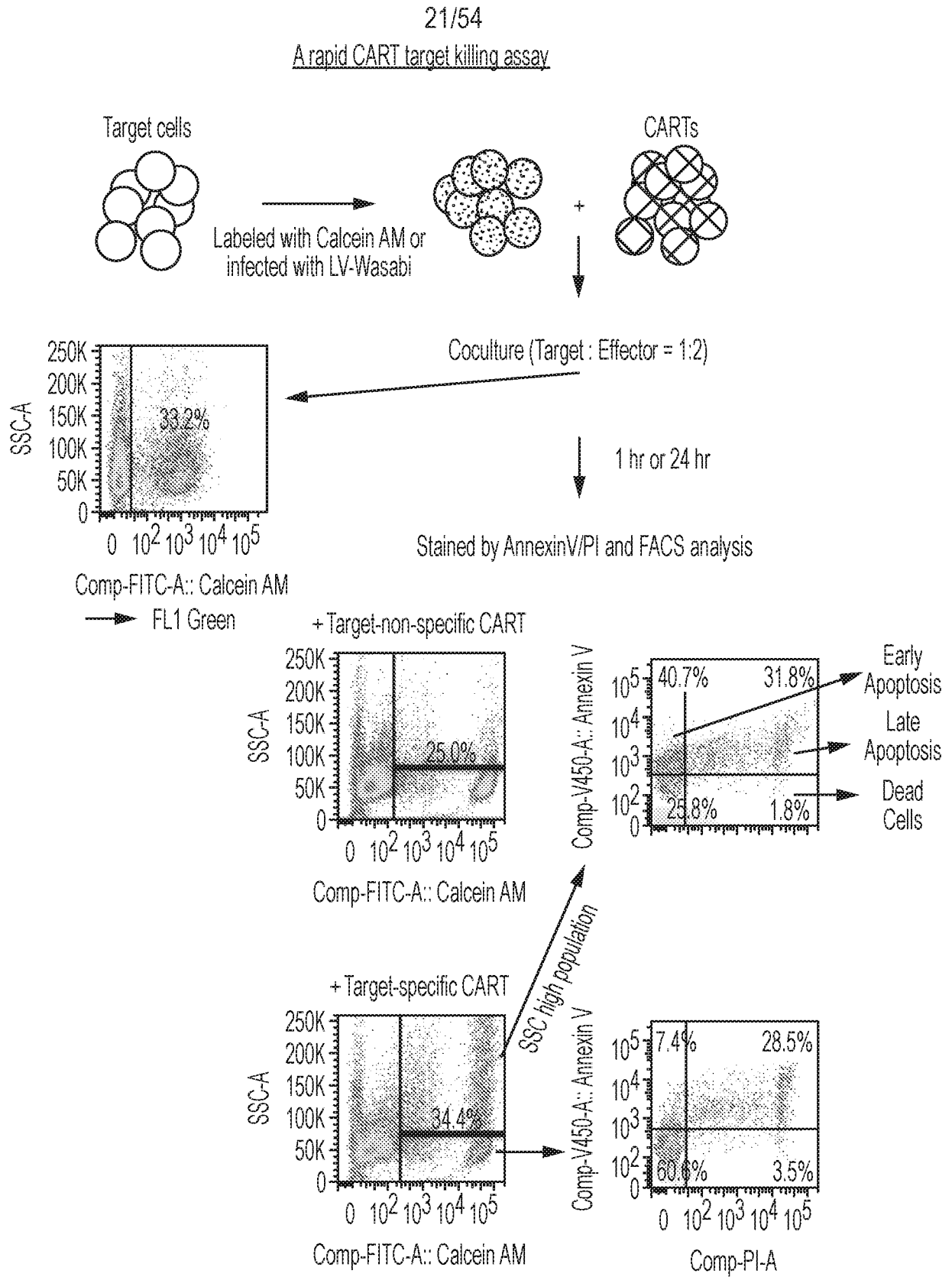
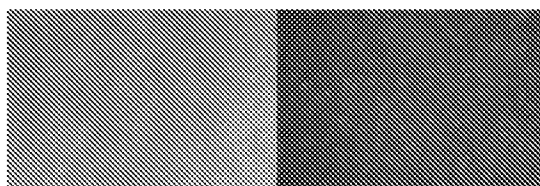


FIG. 14

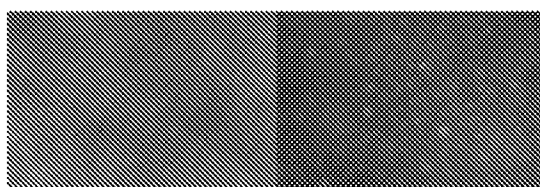
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CD30-CAR T cells targeting primary CD30+ B lymphoma cells
Day 12 under fluorescent microscope

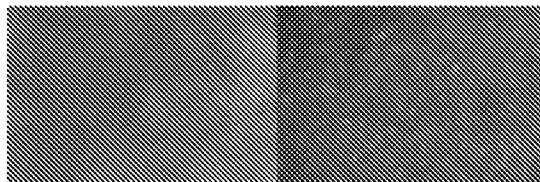
GFP+ B lymphoma



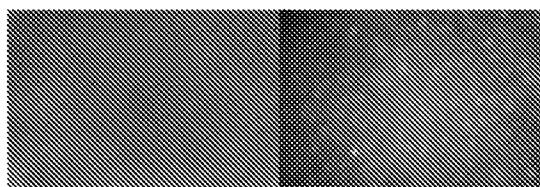
+control T



+GD2-CAR T



+5F11 CD30-CAR T



+AC10 CD30-CAR T

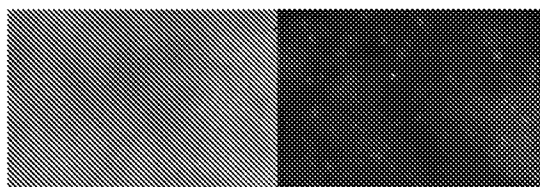


FIG. 15

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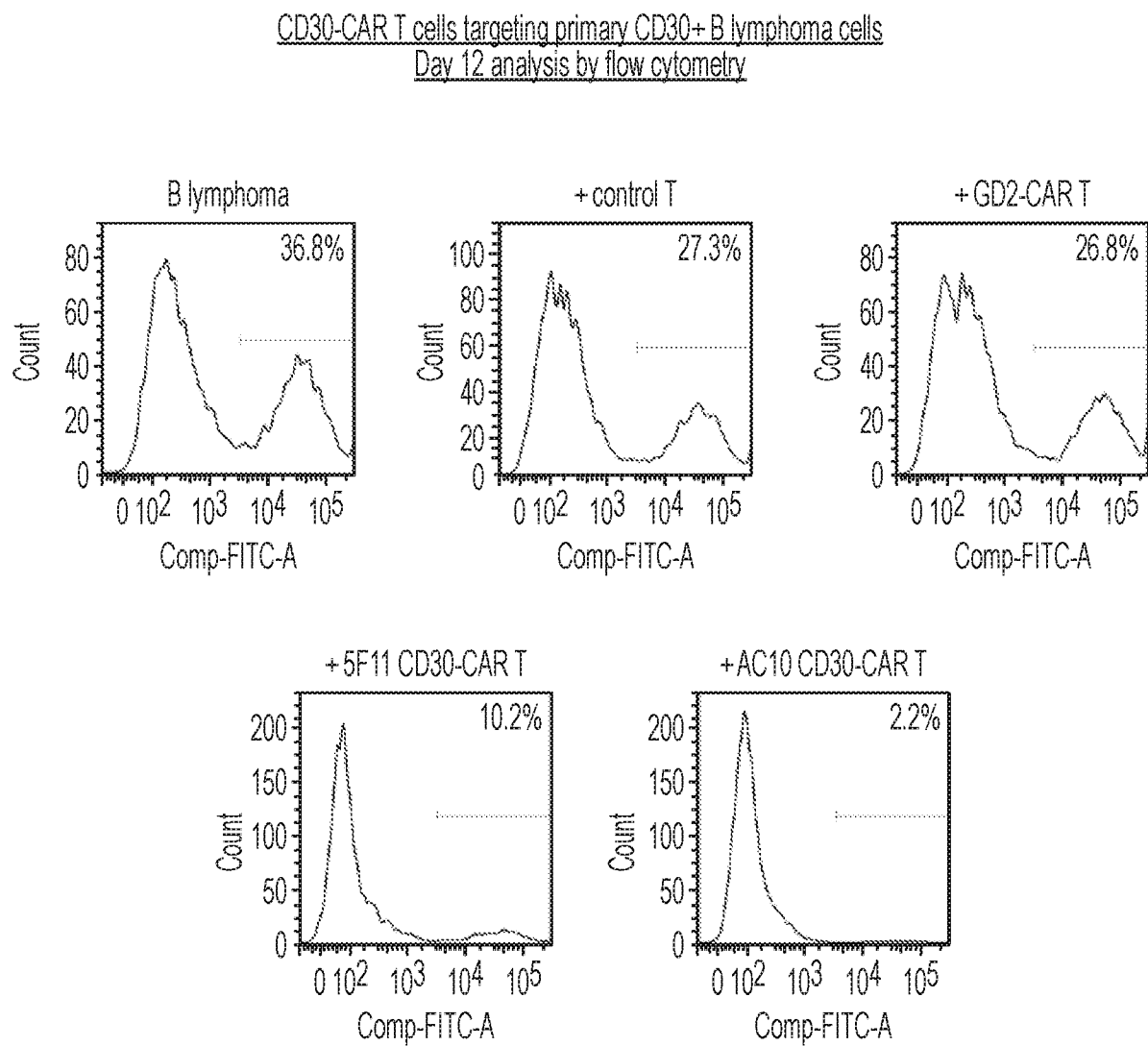


FIG. 16

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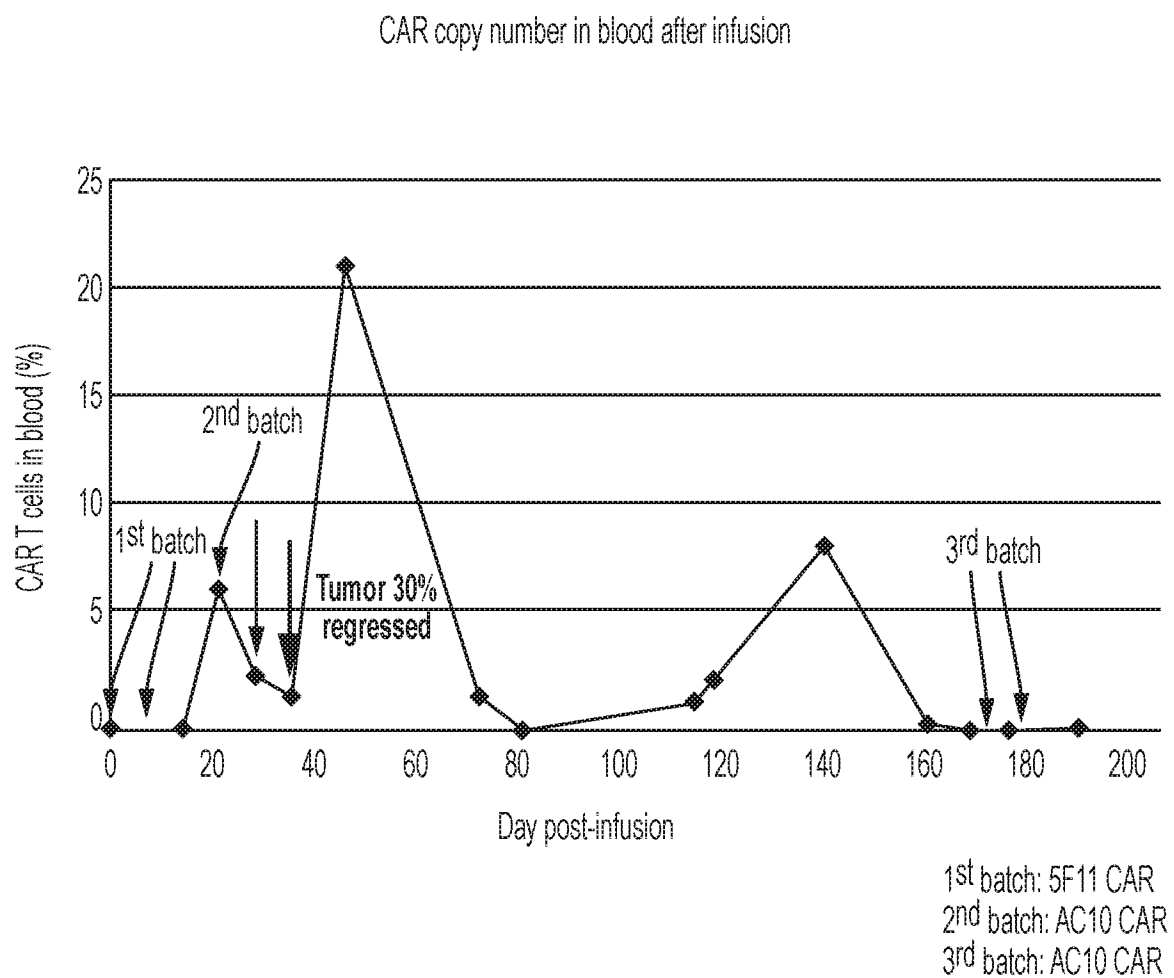
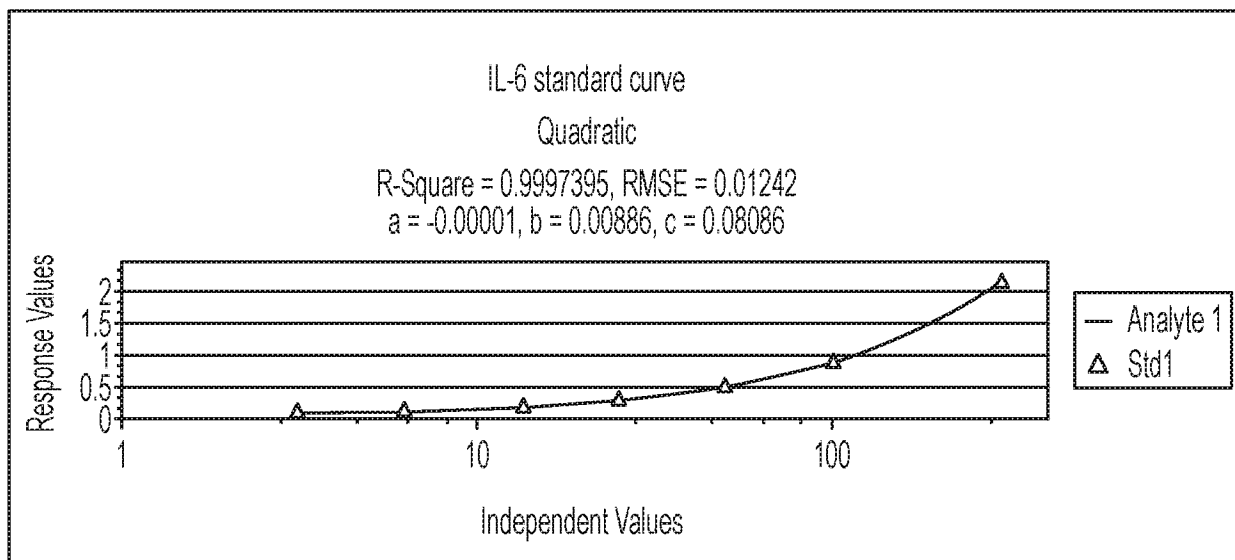


FIG. 17

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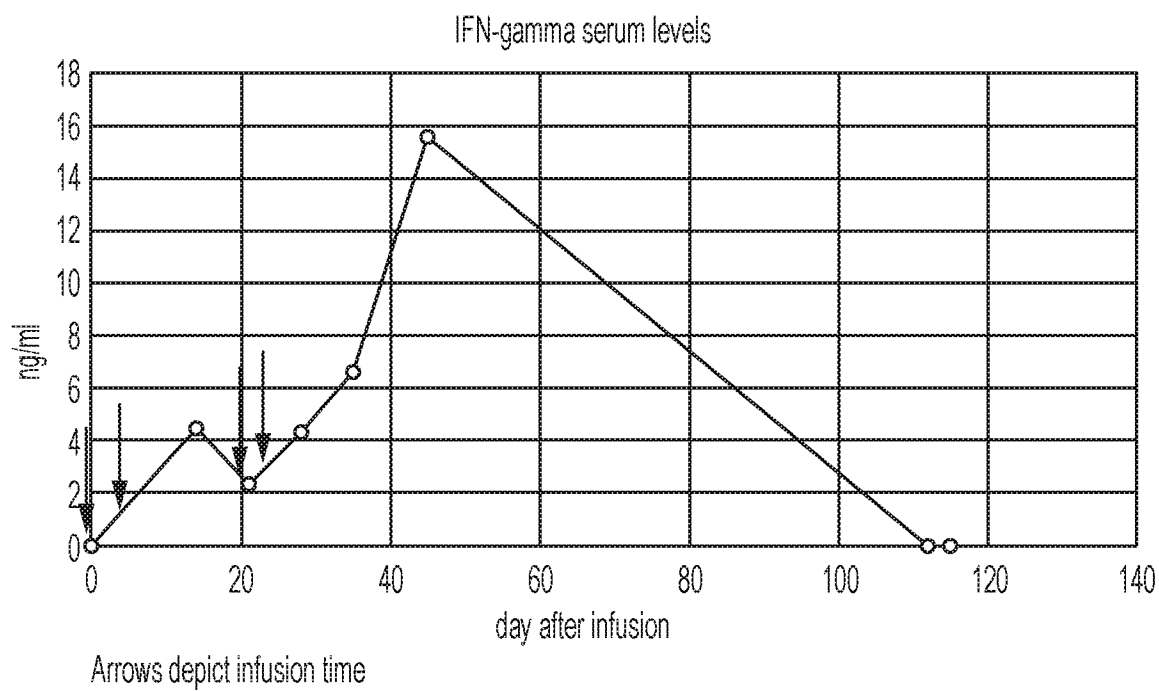
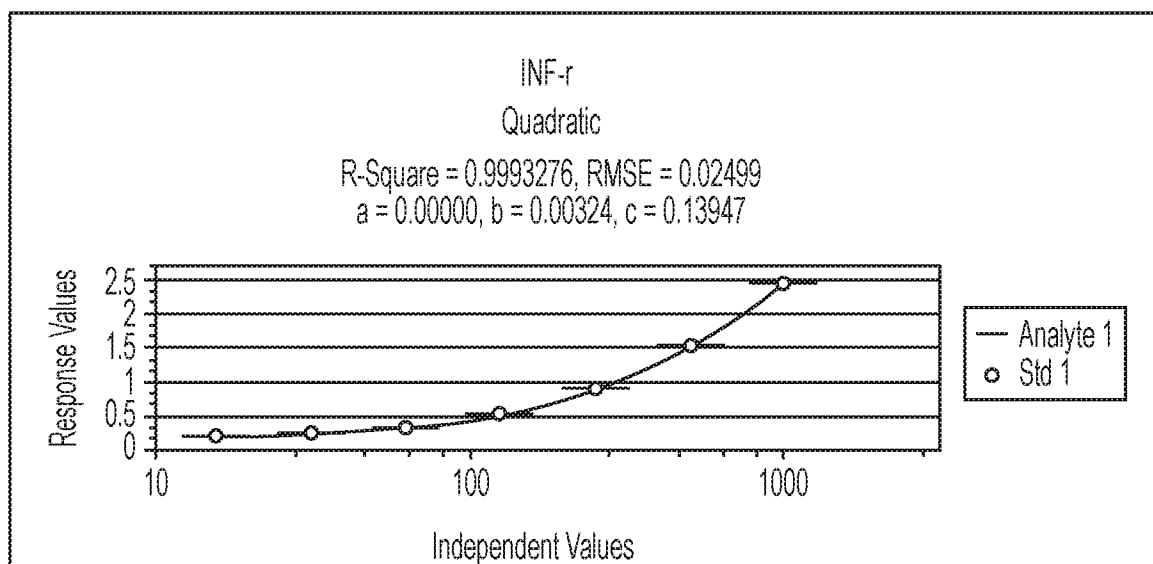


FIG. 19

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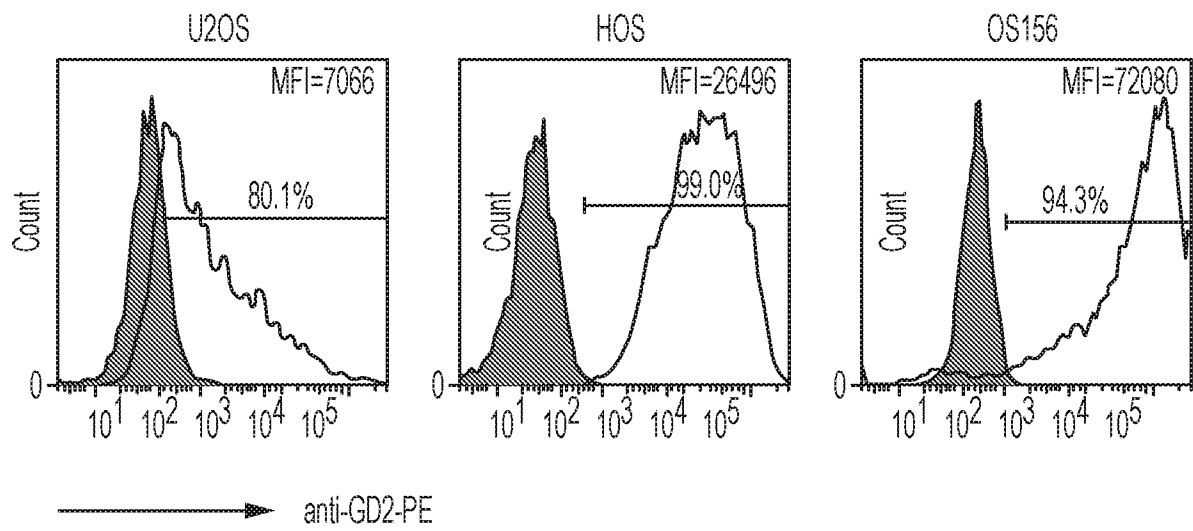


FIG. 20A

FIG. 20B

FIG. 20C

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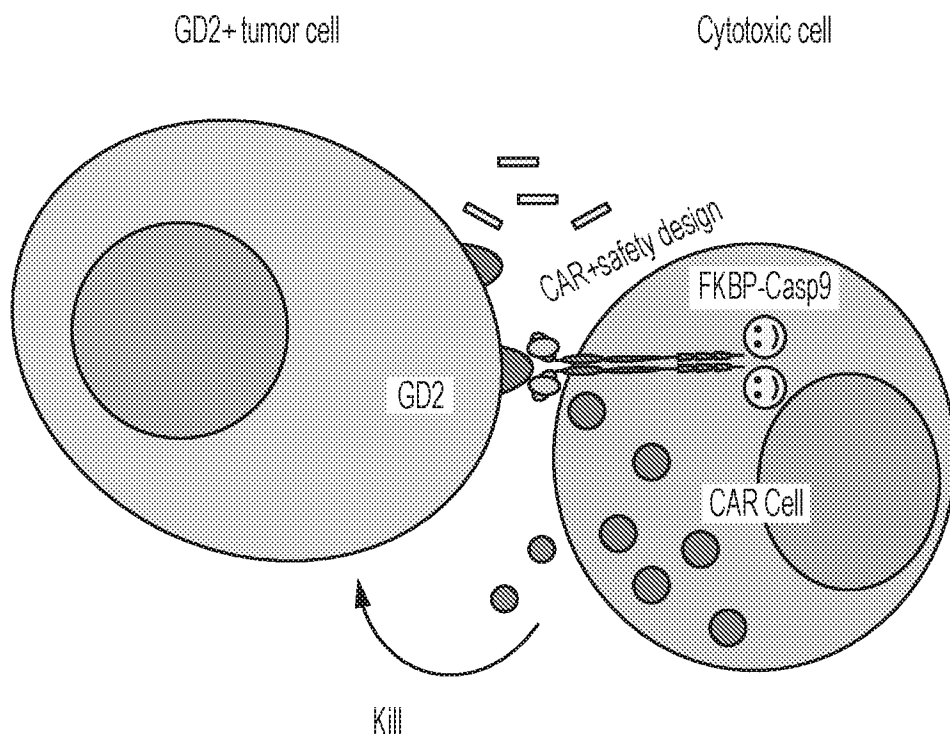
4th generation CAR design targeting GD2-positive tumor

FIG. 21A

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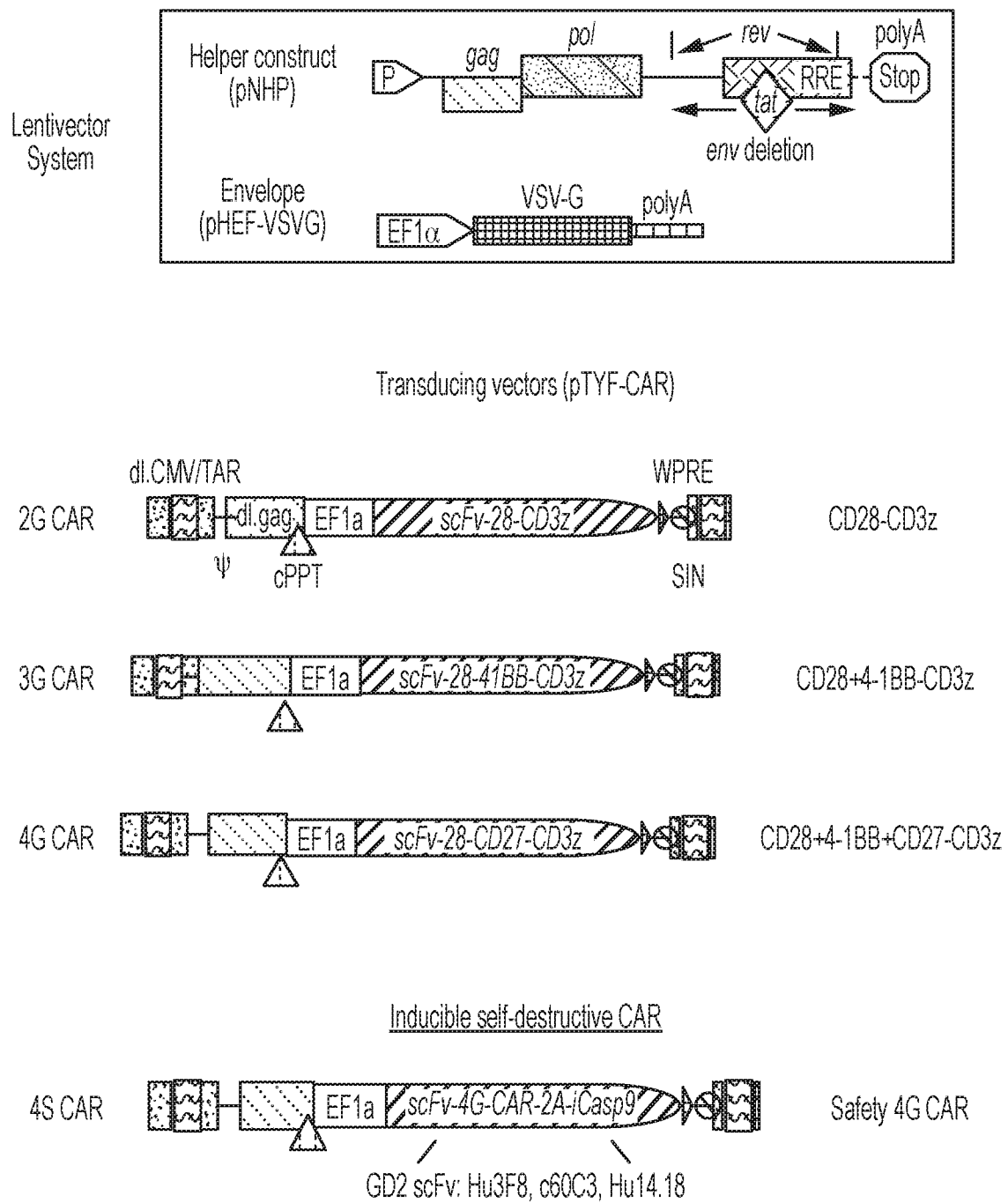
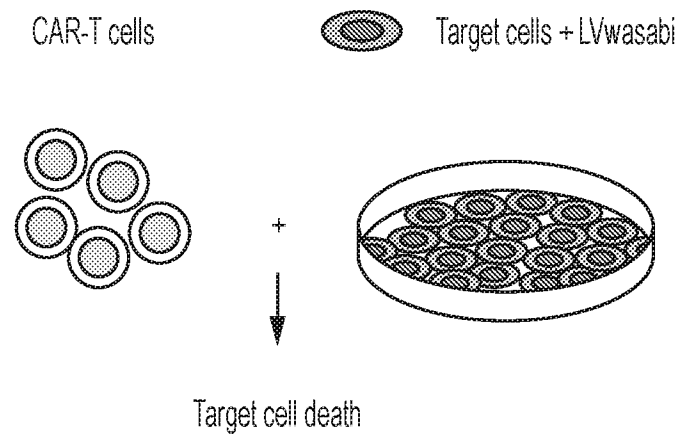
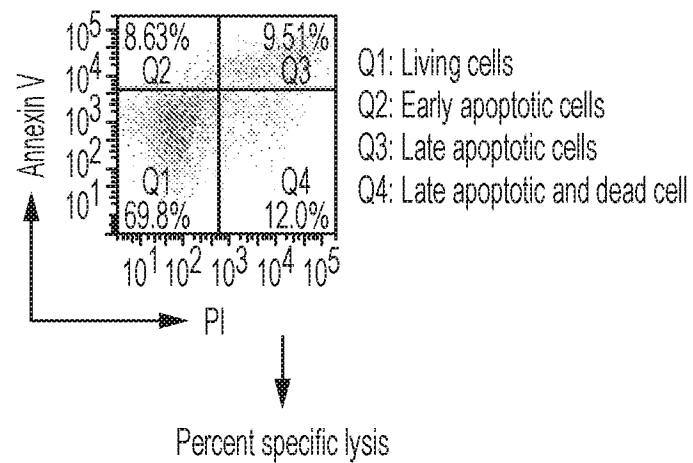


FIG. 21B

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FACS analysis: Annexin V / PI staining



$$\% \text{ specific lysis} = \frac{(\% \text{ apoptotic target} - \% \text{ spontaneous apoptotic cell})}{(100\% - \% \text{ spontaneous apoptotic cell})} \times 100$$

FIG. 22

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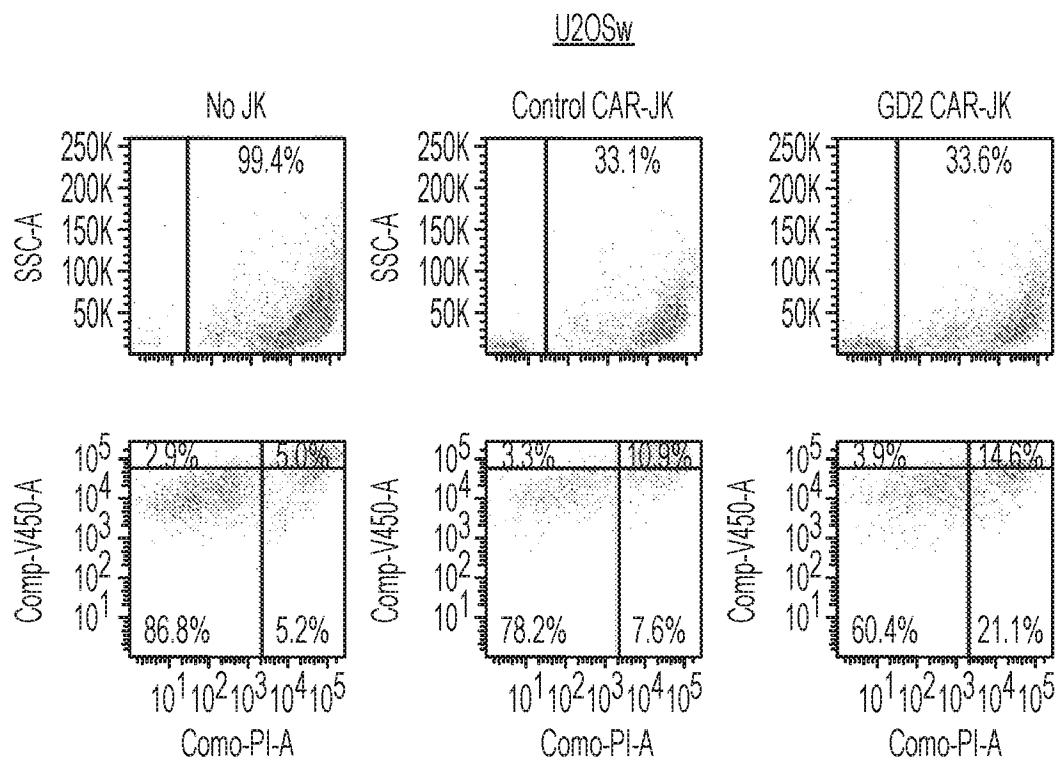


FIG. 23A

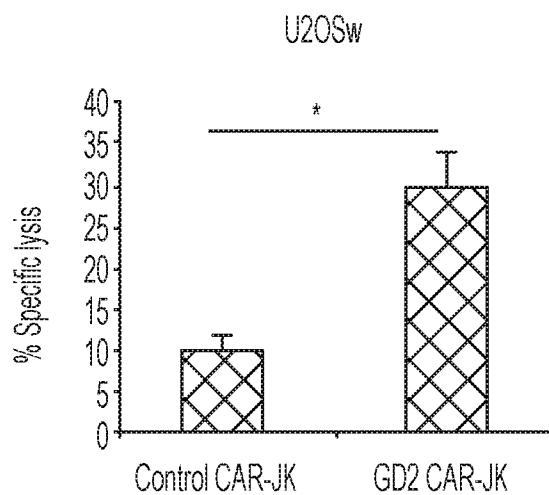


FIG. 23B

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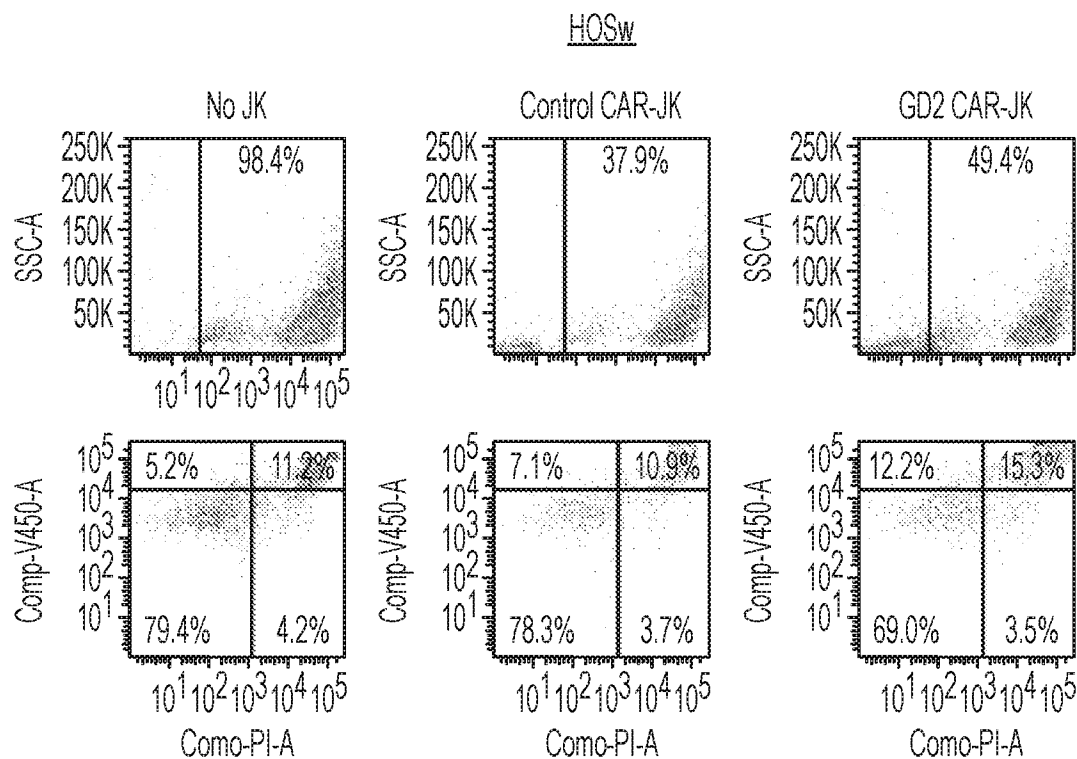


FIG. 23C

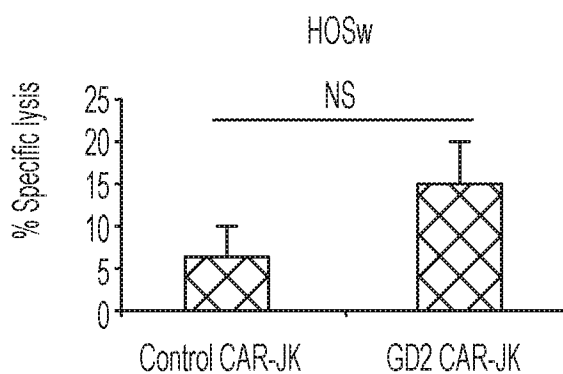


FIG. 23D

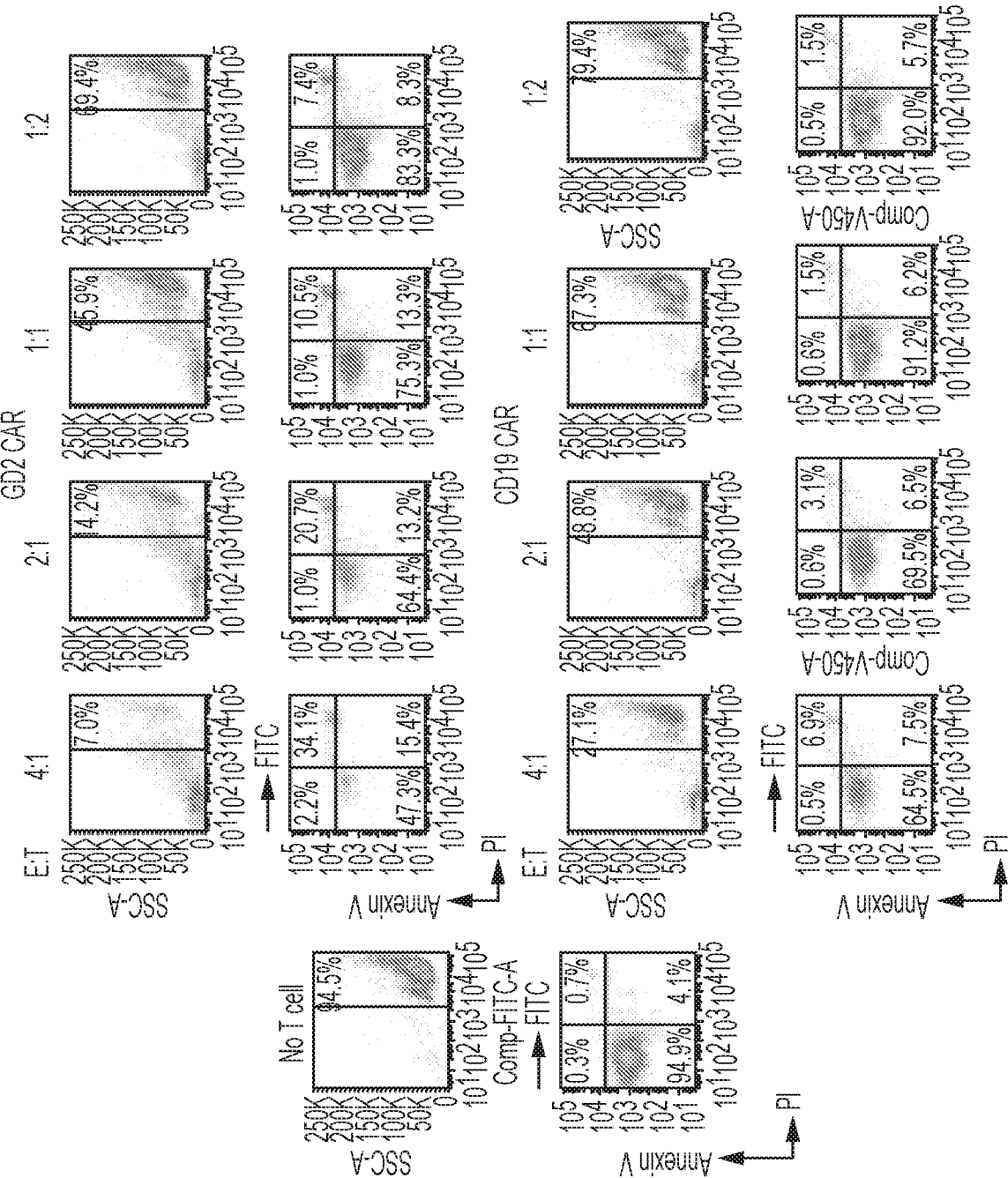


FIG. 24A

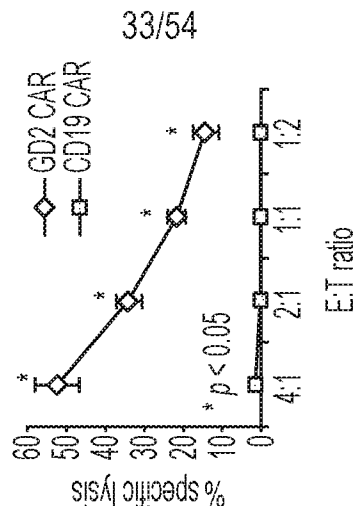


FIG. 24B

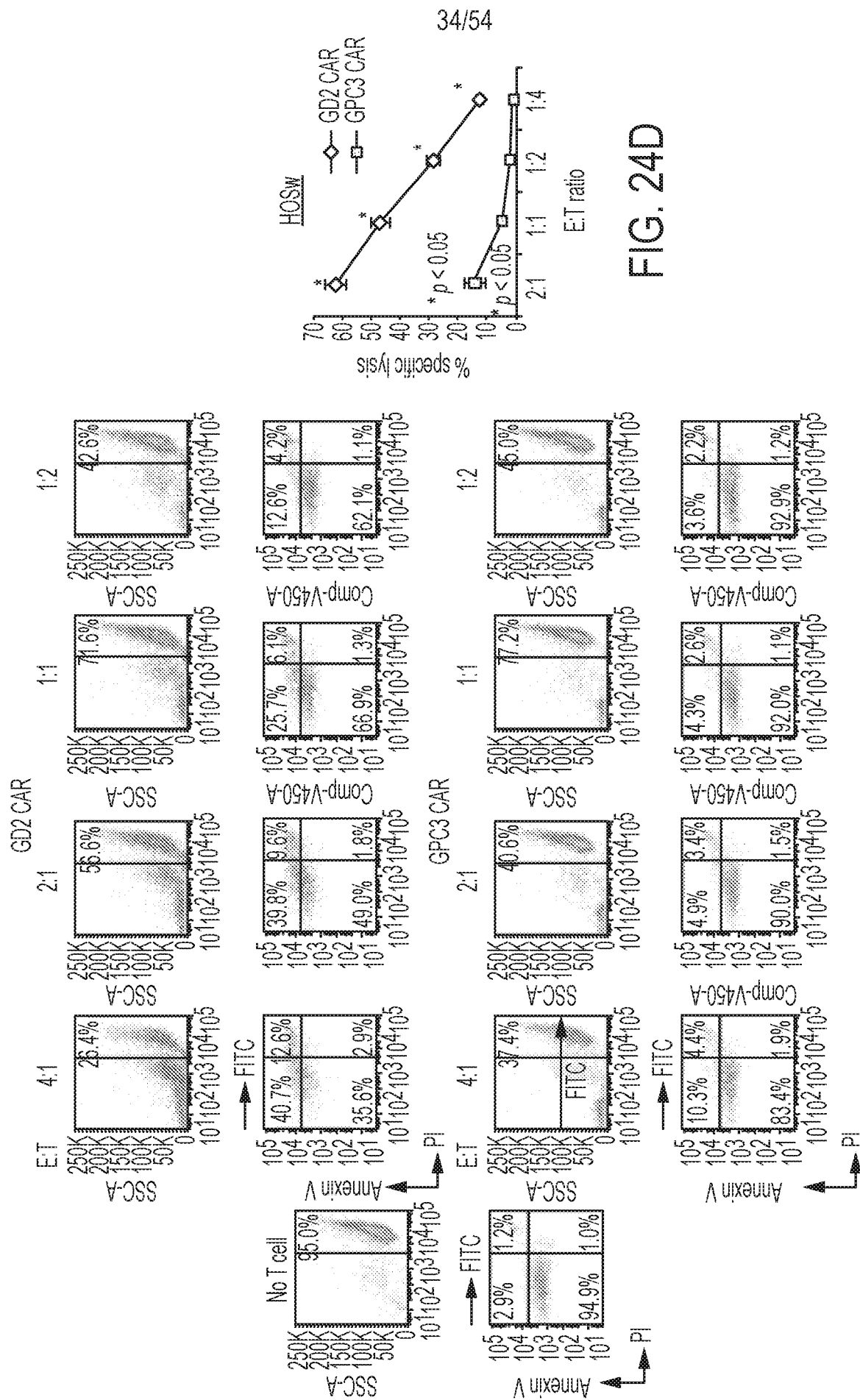


FIG. 24C

FIG. 24D

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ET ratio	GD2 CAR (% specific lysis)	GPC3 CAR (% specific lysis)
2:1	~62*	~10*
1:1	~48*	~5*
1:2	~32*	~1*
1:4	~18*	~0.5*

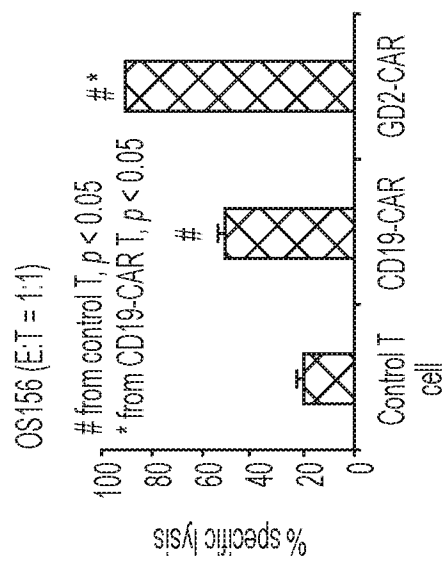


FIG. 24E

OS156

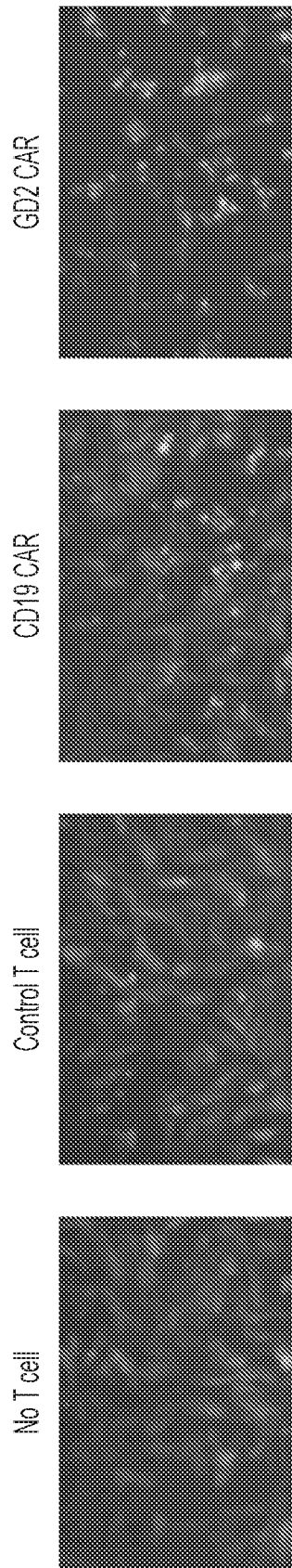


FIG. 24F

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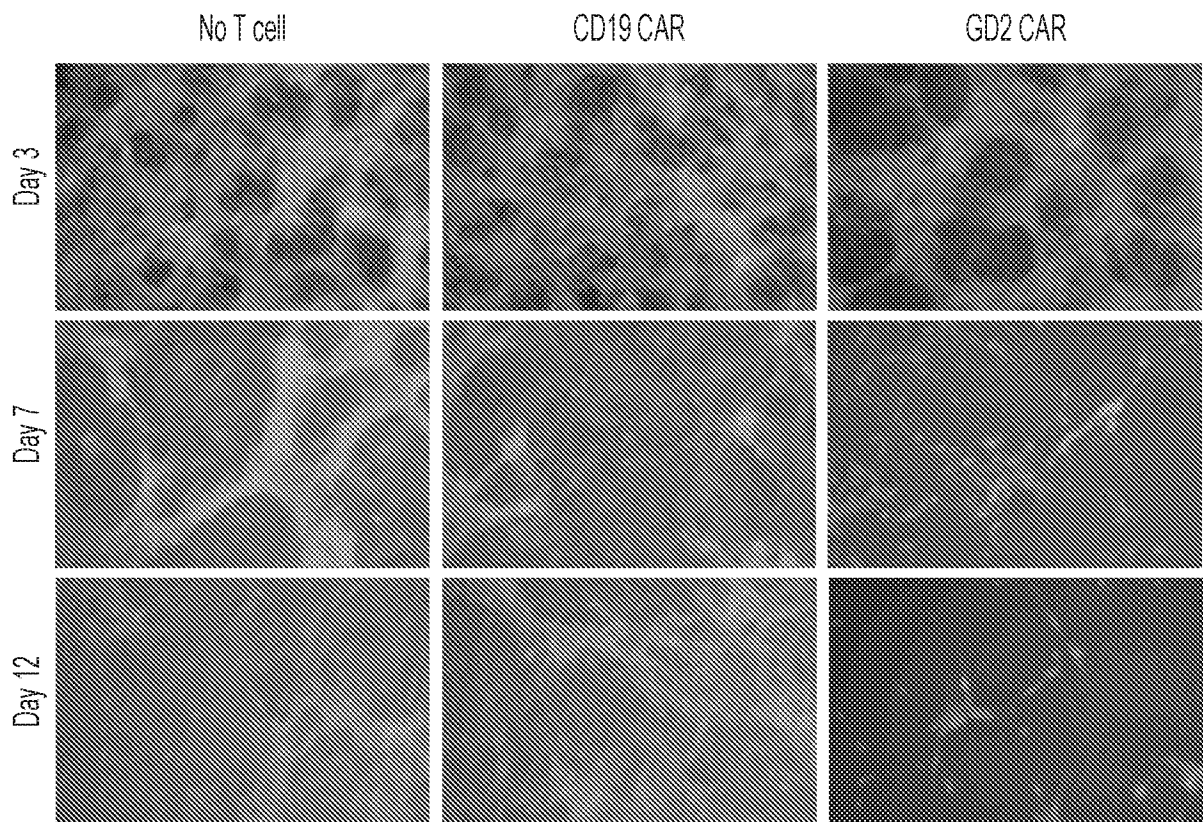


FIG. 24G

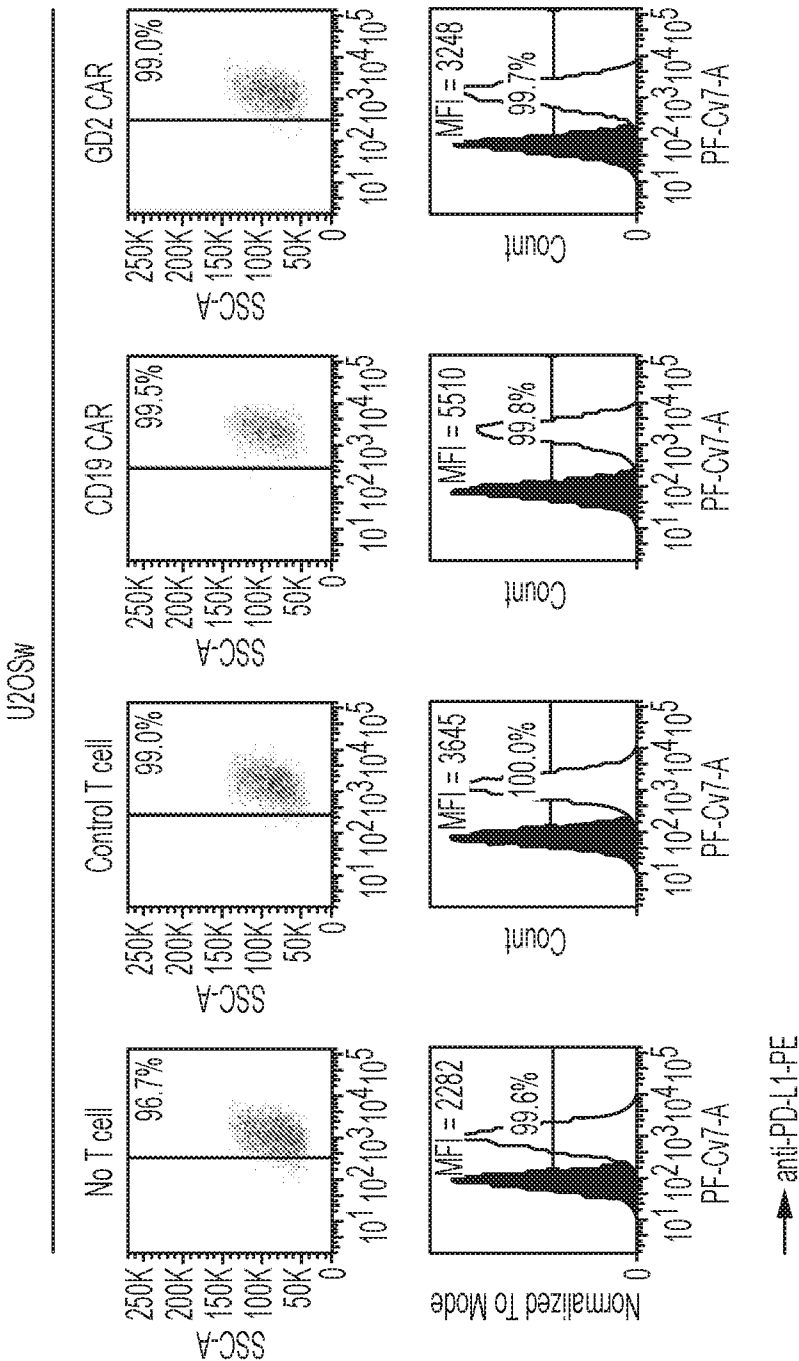


FIG. 25A

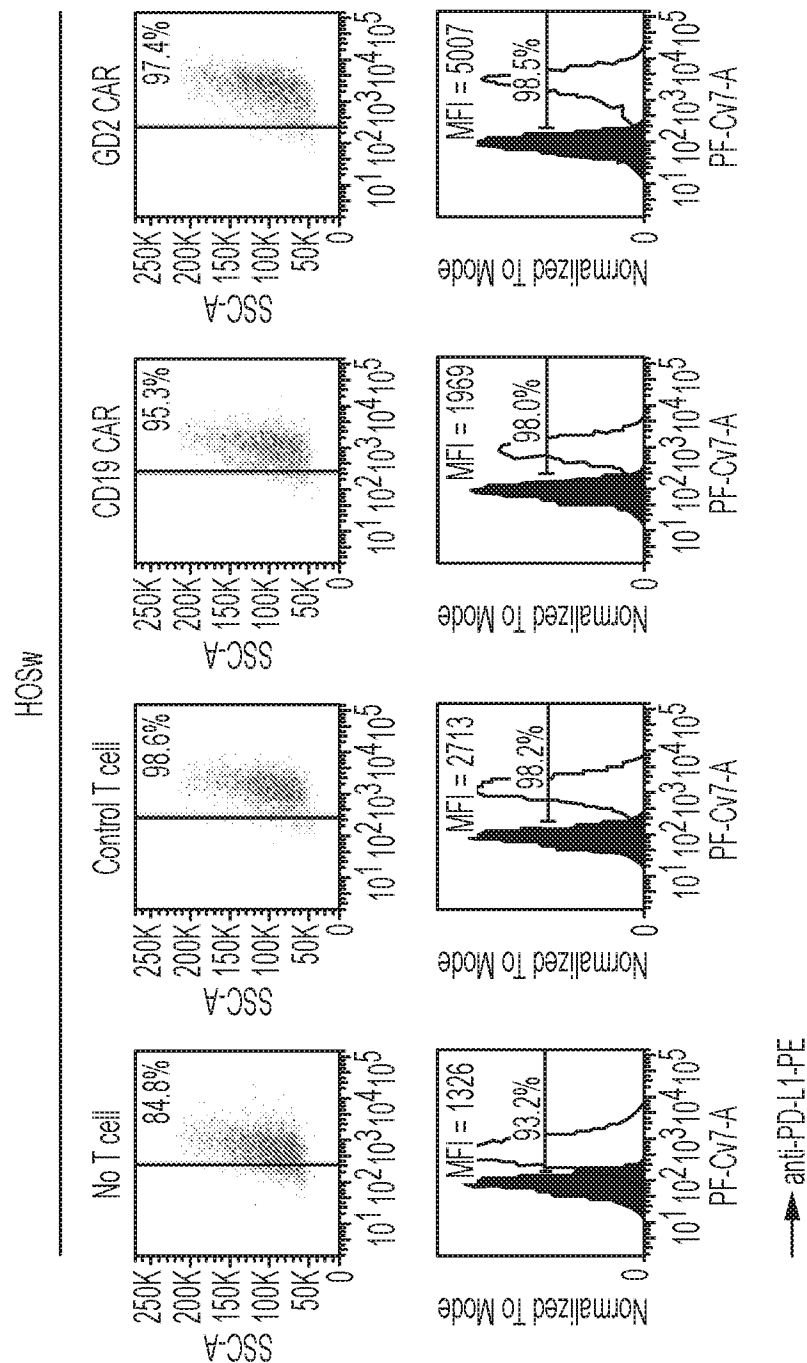


FIG. 25B

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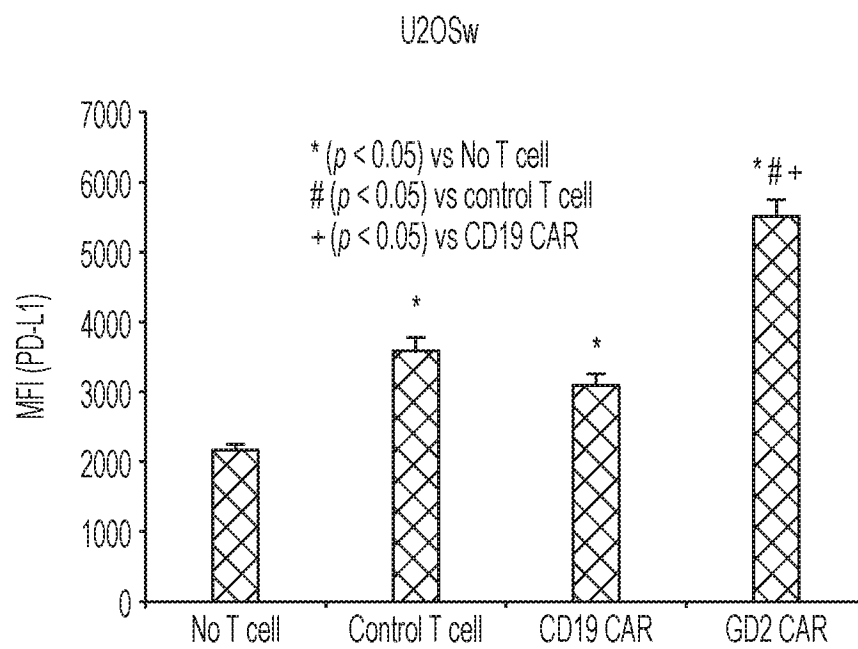


FIG. 25C

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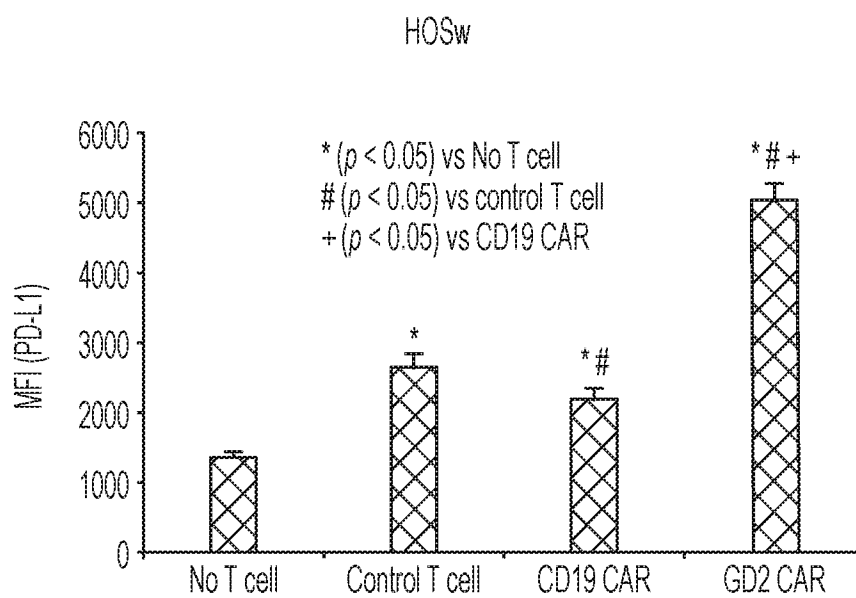


FIG. 25D

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CAR T PD-1 up-regulation + target cells
(subtracted background PD-1 expression)

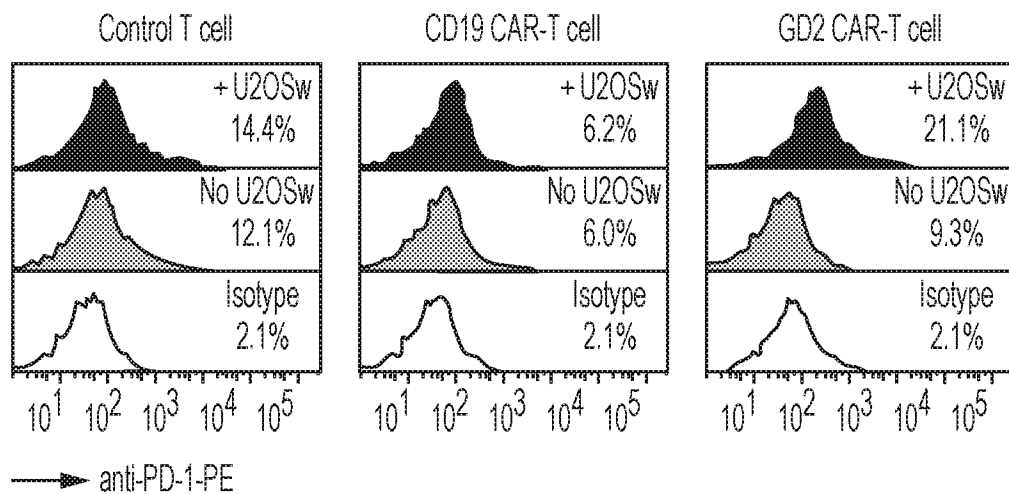
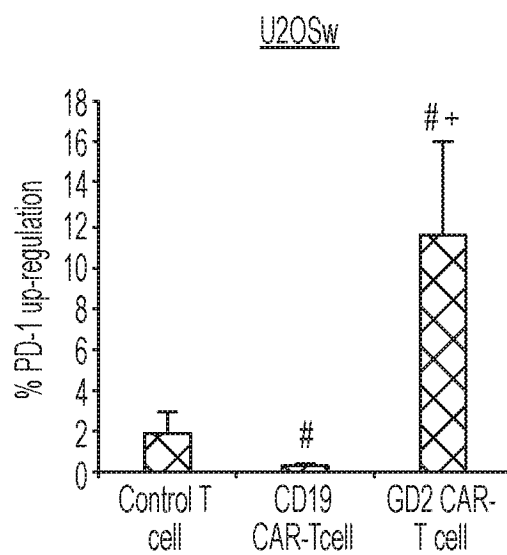


FIG. 26A

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$p < 0.05$ vs no CAR T
+ $p < 0.05$ vs CD19 CAR T

FIG. 26B

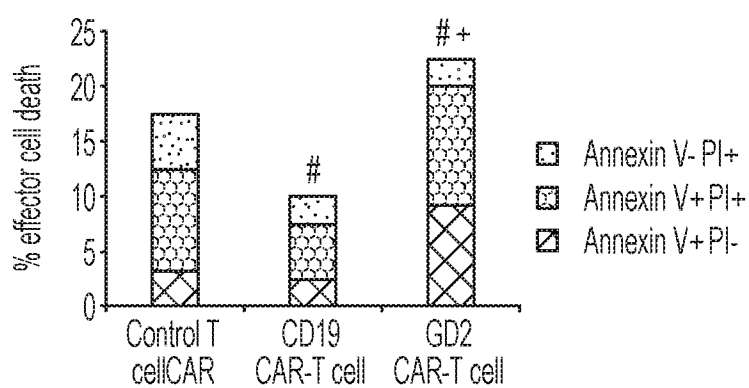


FIG. 26C

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CAR T PD-1 up-regulation + target cells
(subtracted background PD-1 expression)

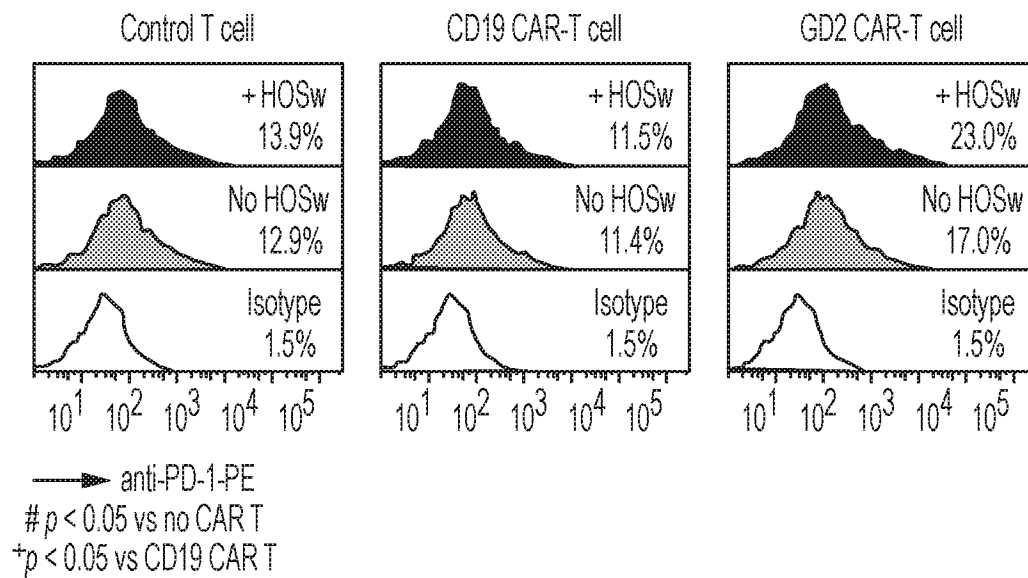


FIG. 26D

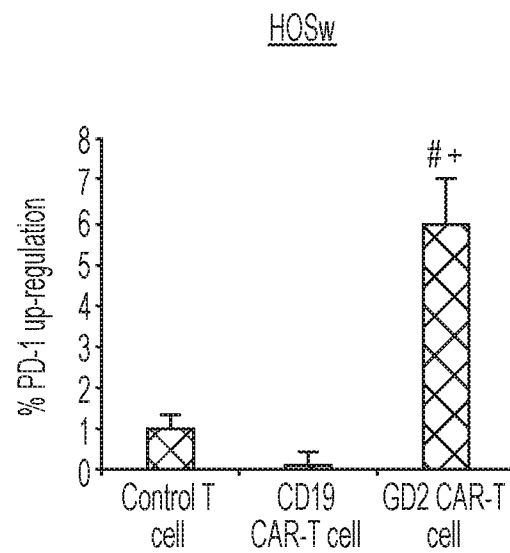


FIG. 26E

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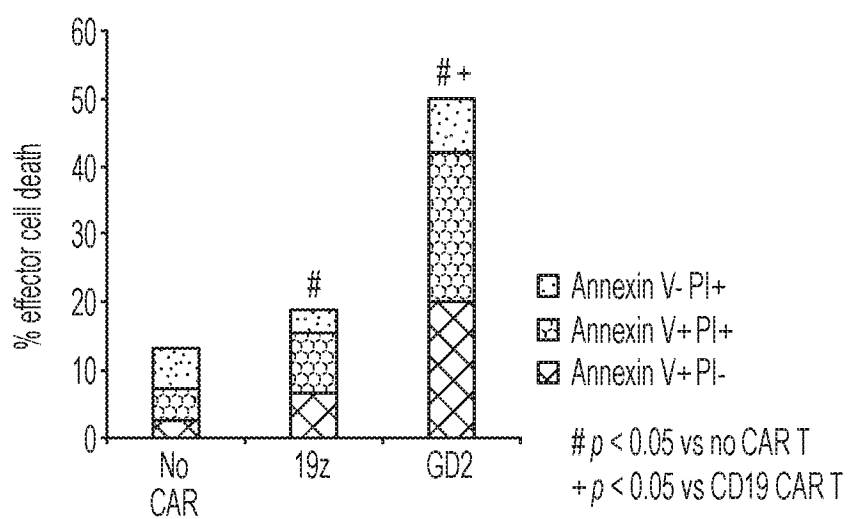


FIG. 26F

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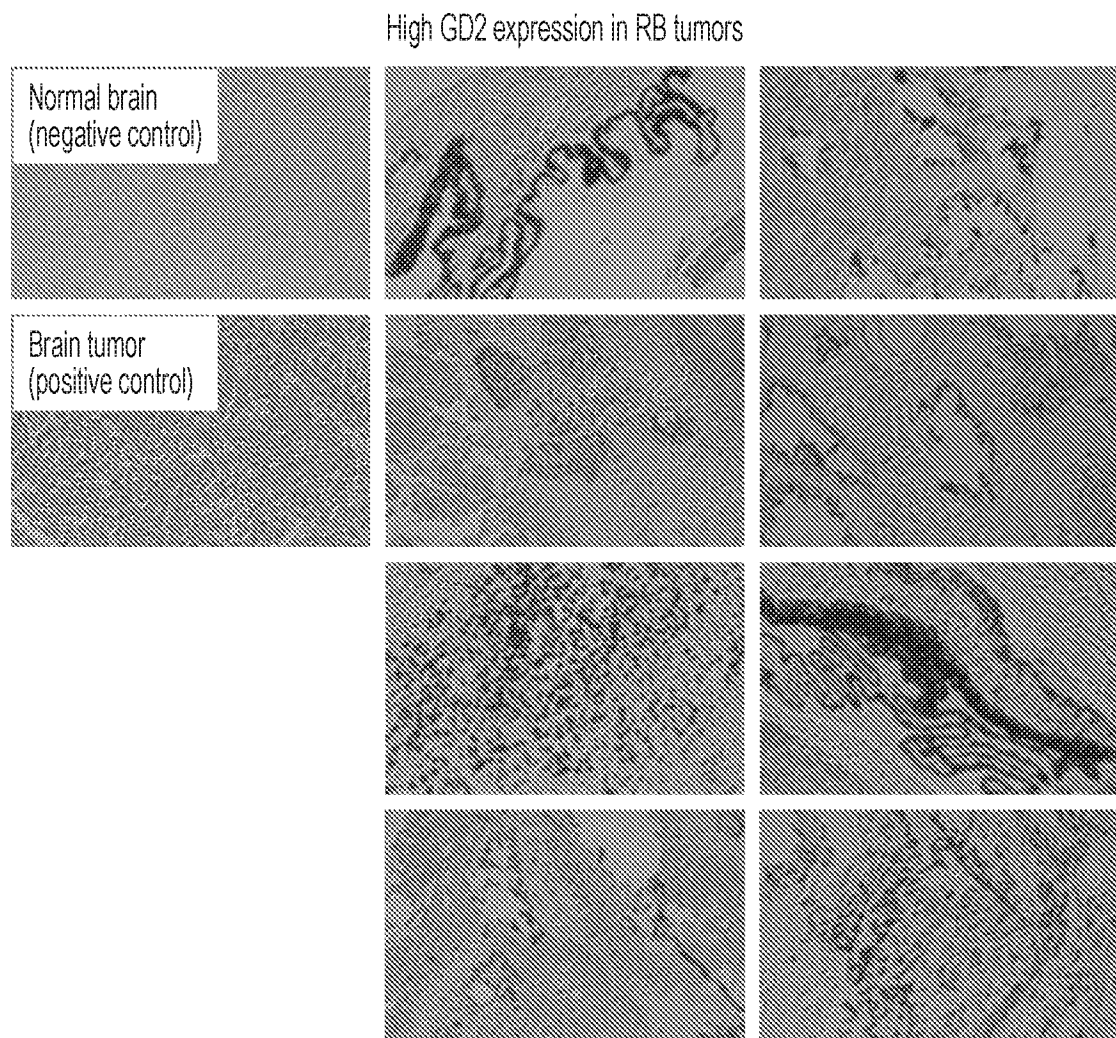


FIG. 27A

GD2 expression in RB (retinoblastoma) versus NB (neuroblastoma)

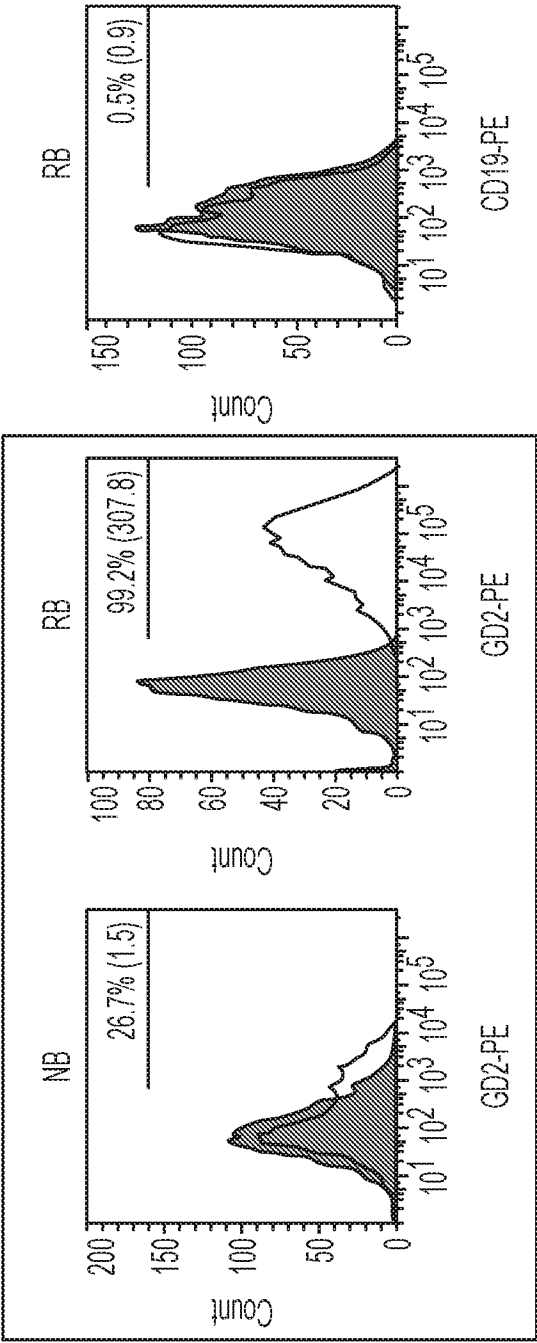


FIG. 27B

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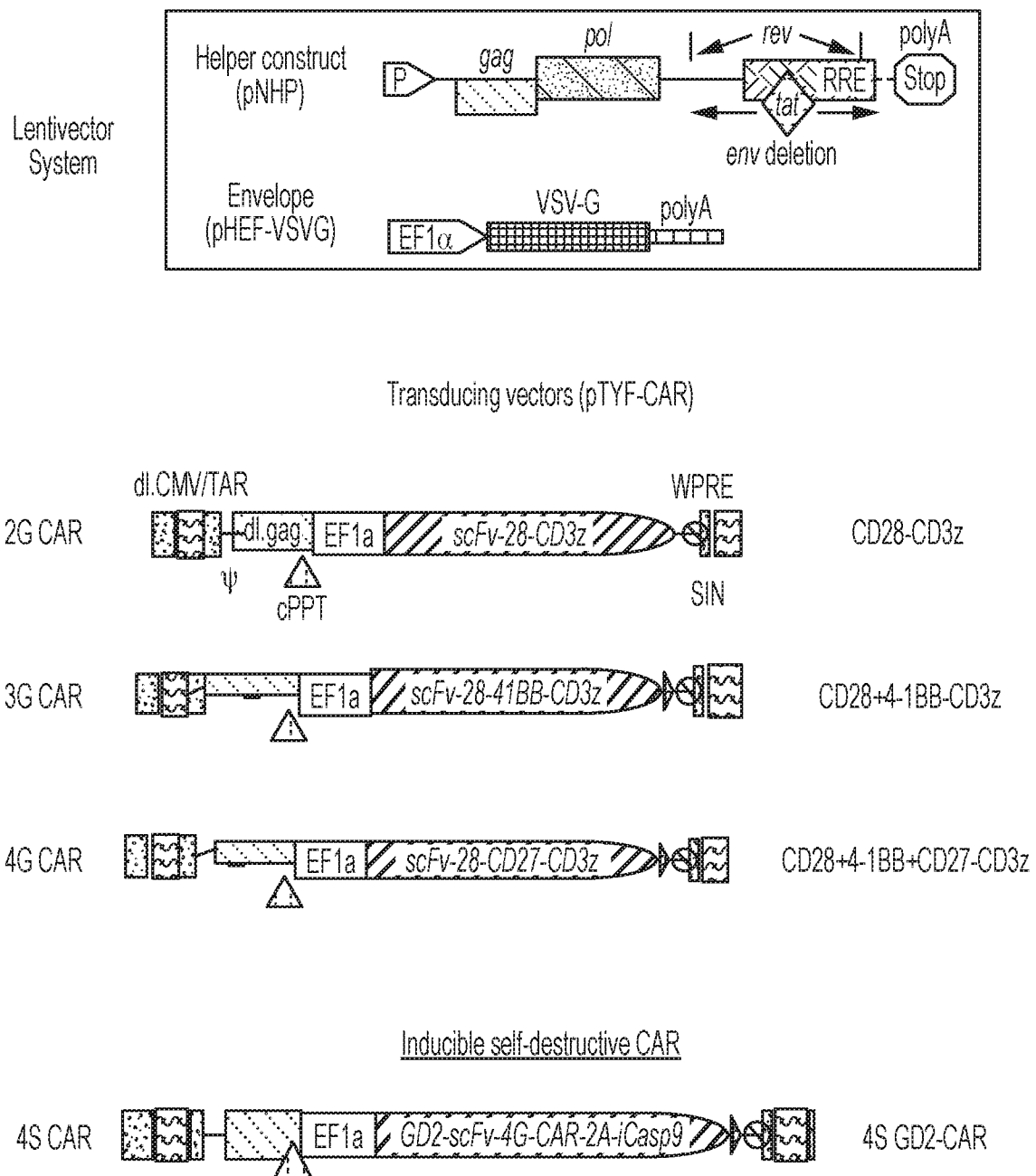


FIG. 28A

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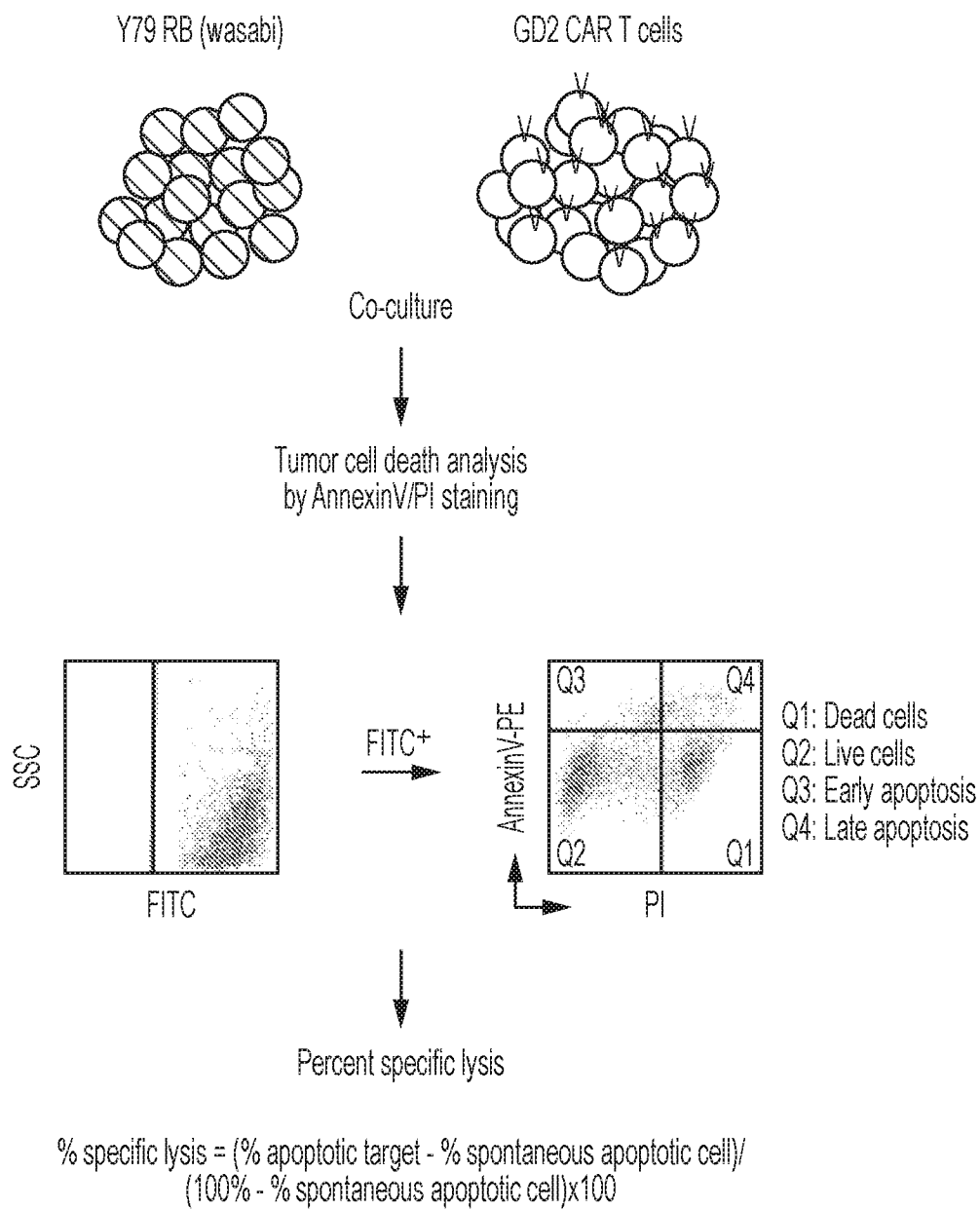


FIG. 28B

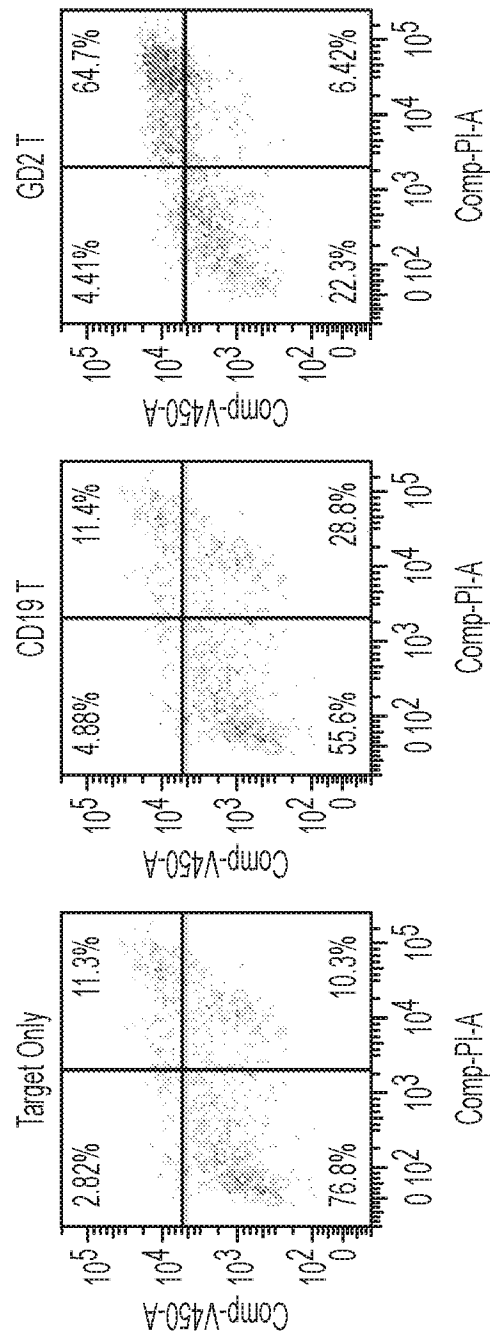


FIG. 29A

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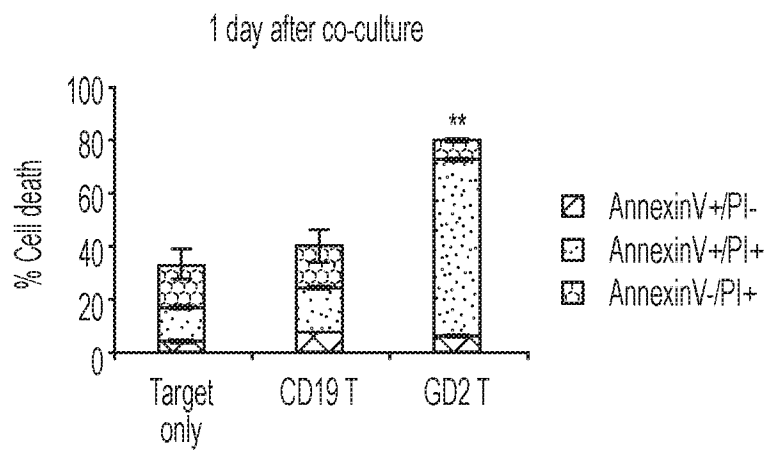


FIG. 29B

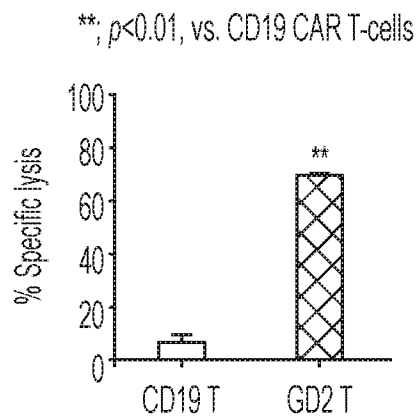


FIG. 29C

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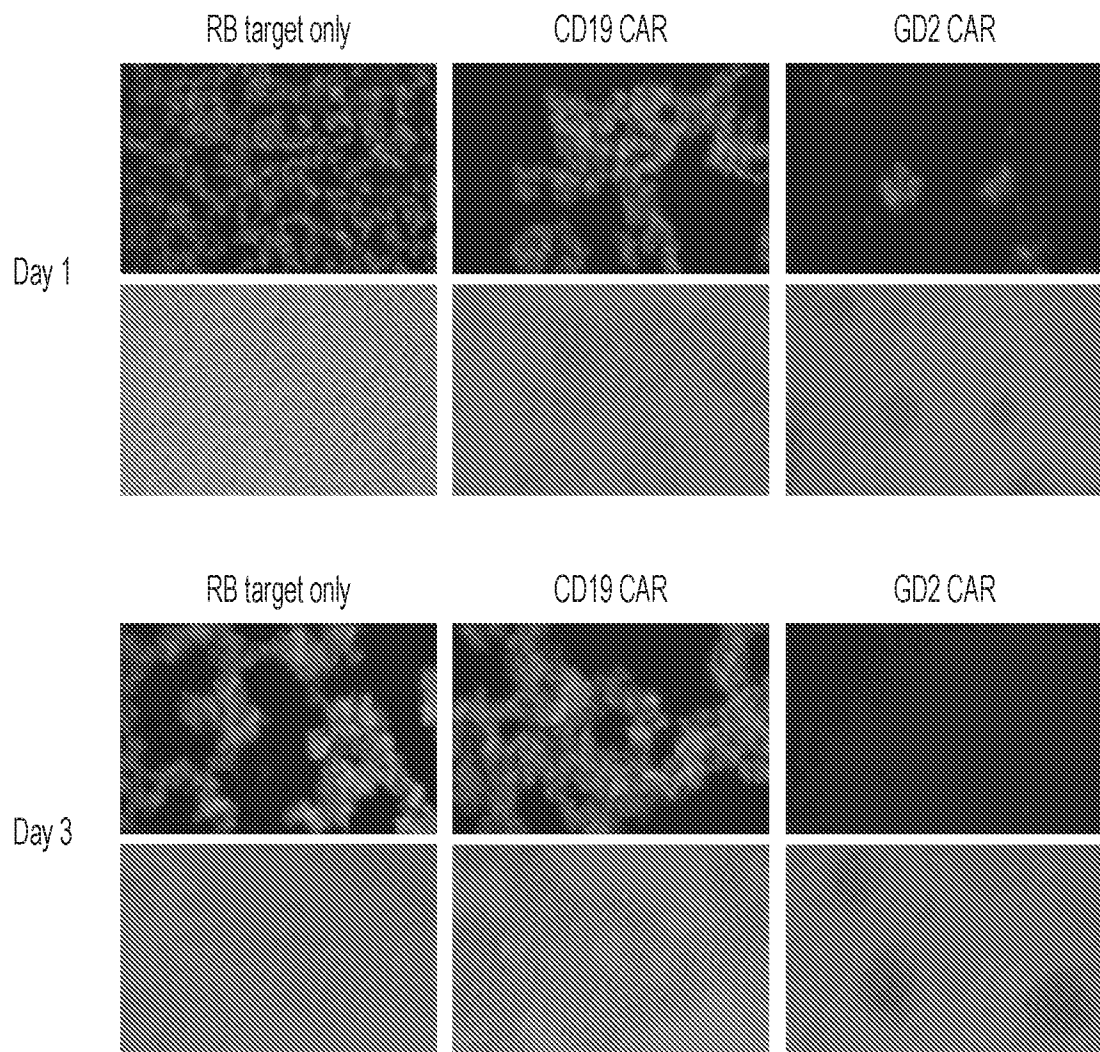


FIG. 29D

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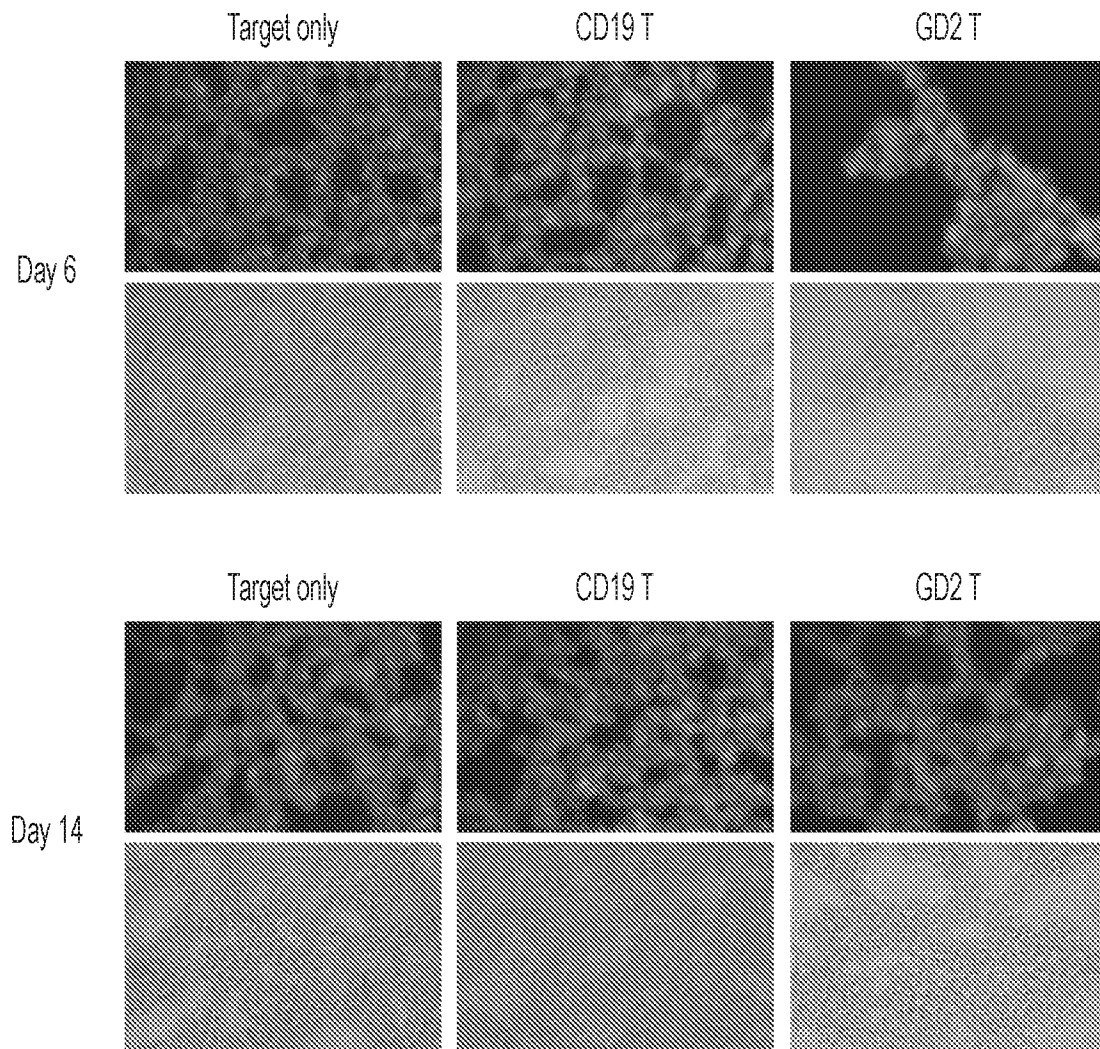
2nd round killing of target RB by GD2-CAR T cells

FIG. 30

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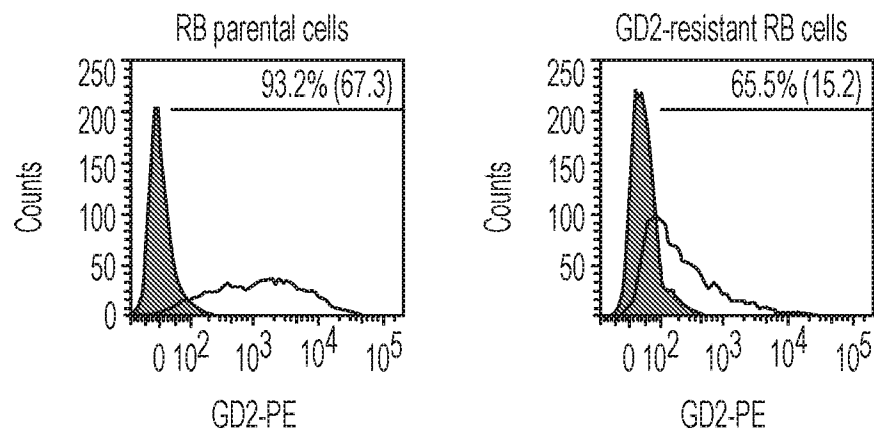
Down-regulation of GD2 in RB tumor upon GD2-CAR T targeting

FIG. 31A

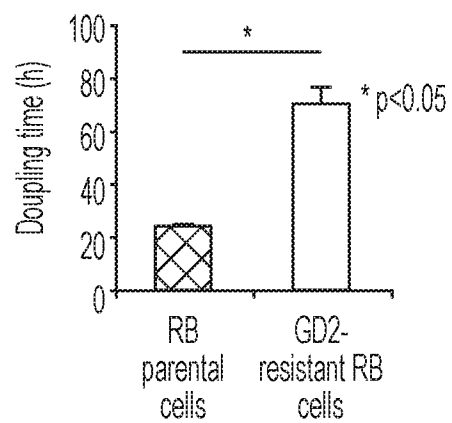


FIG. 31B

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Up-regulation of PD-L1 and down-regulation of GD2 of RB tumor upon GD2-CAR T cell targeting

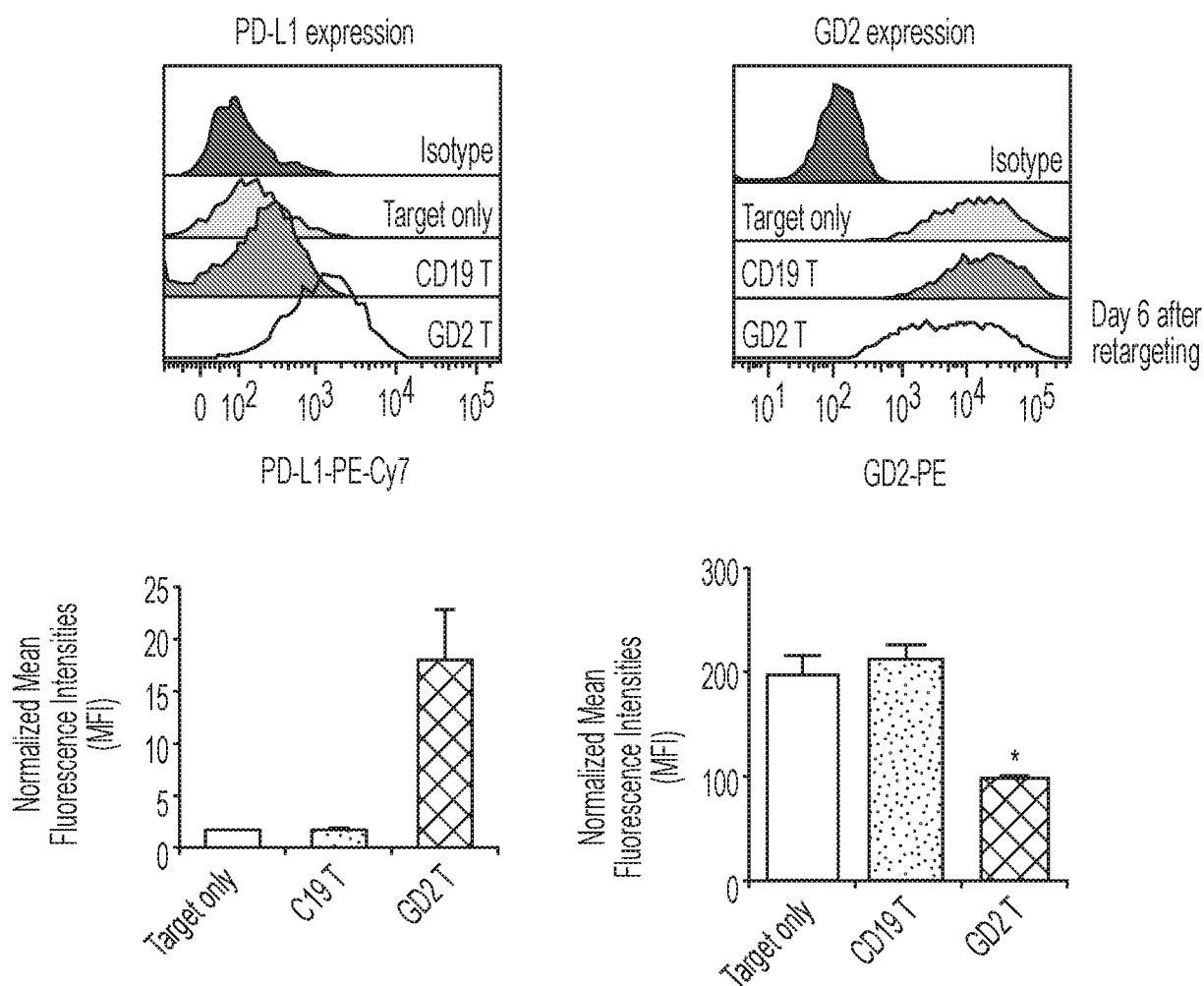


FIG. 31C

FIG. 31D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/018716

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/00; C12N 15/09; C12N 15/85 (2016.01)

CPC - C07K 14/7051; C07K 2317/00; C07K 2317/622; C07K 2319/00; C12N 15/00; C12N 15/09 (2016.02)

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)

IPC(8) - C12N 15/00; C12N 15/09; C12N 15/85 (2016.01)

CPC - C07K 14/7051; C07K 2317/00; C07K 2317/622; C07K 2319/00; C12N 15/00; C12N 15/09; C12N 15/85; C12N 2510/00 (2016.02)

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

USPC 435/69.1; 536/23.1; 536/23.5 (keyword delimited)

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

PatBase, Orbit, Google Patents, Google Scholar, Google

Search terms used: IL-15 receptor alpha/IL-15Ra; CD28; CD3zeta/CD247; CAR/chimeric antigen receptor; CD8; CD27; GD2; osteosarcoma/retinoblastoma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/0023937 A1 (BAYLOR COLLEGE OF MEDICINE) 22 January 2015 (22.01.2015) entire document	20-22
X	WO 2014/165707 A2 (MEMORIAL SLOAN-KETTERING CANCER CENTER) 09 October 2014 (09.10.2014) entire document	43-45
X	HIGHFILL et al. "Anti-PD1 Therapy for Pediatric Sarcomas" 27 Mar 2014 (27.03.2014). [Retrieved on 10 May 2016] Retrieved from the internet. <http://sarcomahelp.org/research/immunotherapy-pediatric-sarcomas.html#tpm2_1> entire document	60-62
Y	WO 2014/124143 A1 (ANTHROGENESIS CORPORATION) 14 August 2014 (14.08.2014) entire document	1-3
Y	WU et al. "The IL-15 receptor α chain cytoplasmic domain is critical for normal IL-15Ra function but is not required for trans-presentation." Blood. 01 Dec 2008 (01.12.2008); 112(12):4411-9. entire document	1-3
P,X	WO 2015/179801 A1 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.) 26 November 2015 (26.11.2015) entire document	1-3, 20-22, 43-45, 60-62

☐ 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

"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

11 May 2016

Date of mailing of the international search report

17 JUN 2016

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Authorized officer

Blaine R. Copenheaver

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/018716

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.: 4-19, 23-42, 46-59, 63
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.