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Hua et al.

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(54) **COMPOSITIONS AND METHODS FOR TARGETING CD13 AND TIM-3 WITH CAR T CELLS TO TREAT ACUTE MYELOID LEUKEMIA**

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CPC ..... A61K 35/17; A61K 39/4611; A61K 39/4631; A61K 39/464411;

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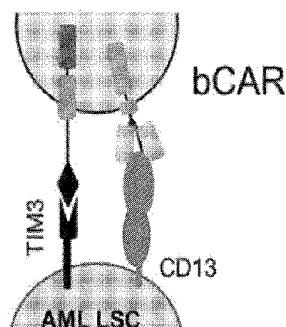
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(57) **ABSTRACT**

The present invention includes compositions and methods for treating AML utilizing bispecific CARs. In certain aspects, the invention includes a bispecific split CAR which binds CD13 and TIM-3 on AML cells.

**15 Claims, 21 Drawing Sheets**

**Specification includes a Sequence Listing.**



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(58)	<b>Field of Classification Search</b>						
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See application file for complete search history.

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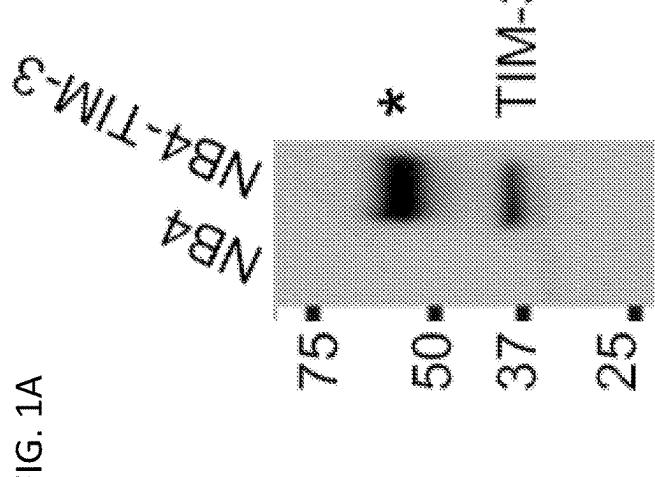


FIG. 1B

FIG. 1C

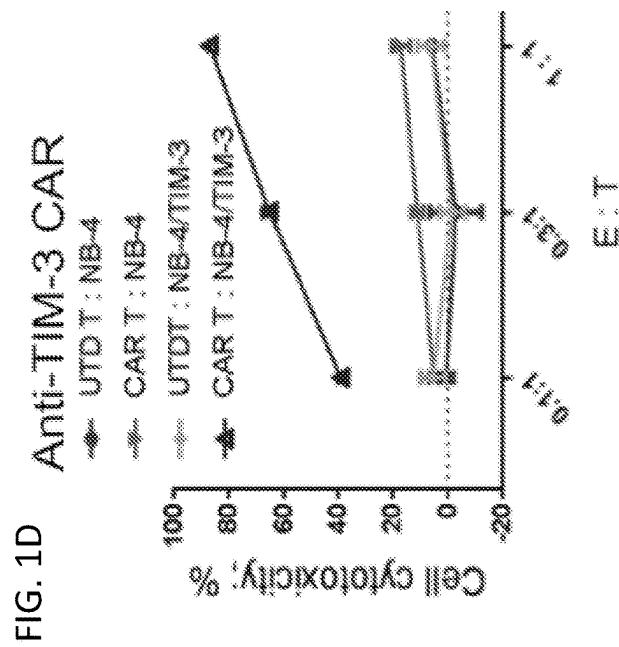
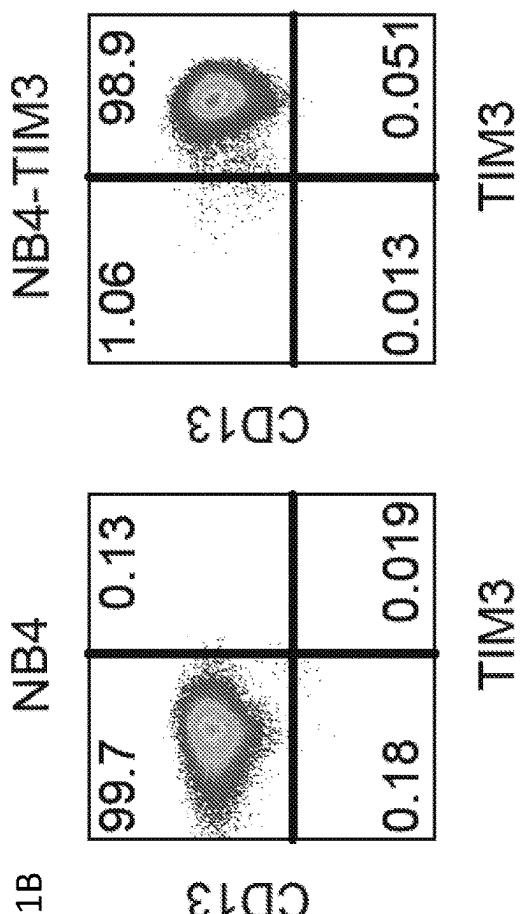


FIG. 1C

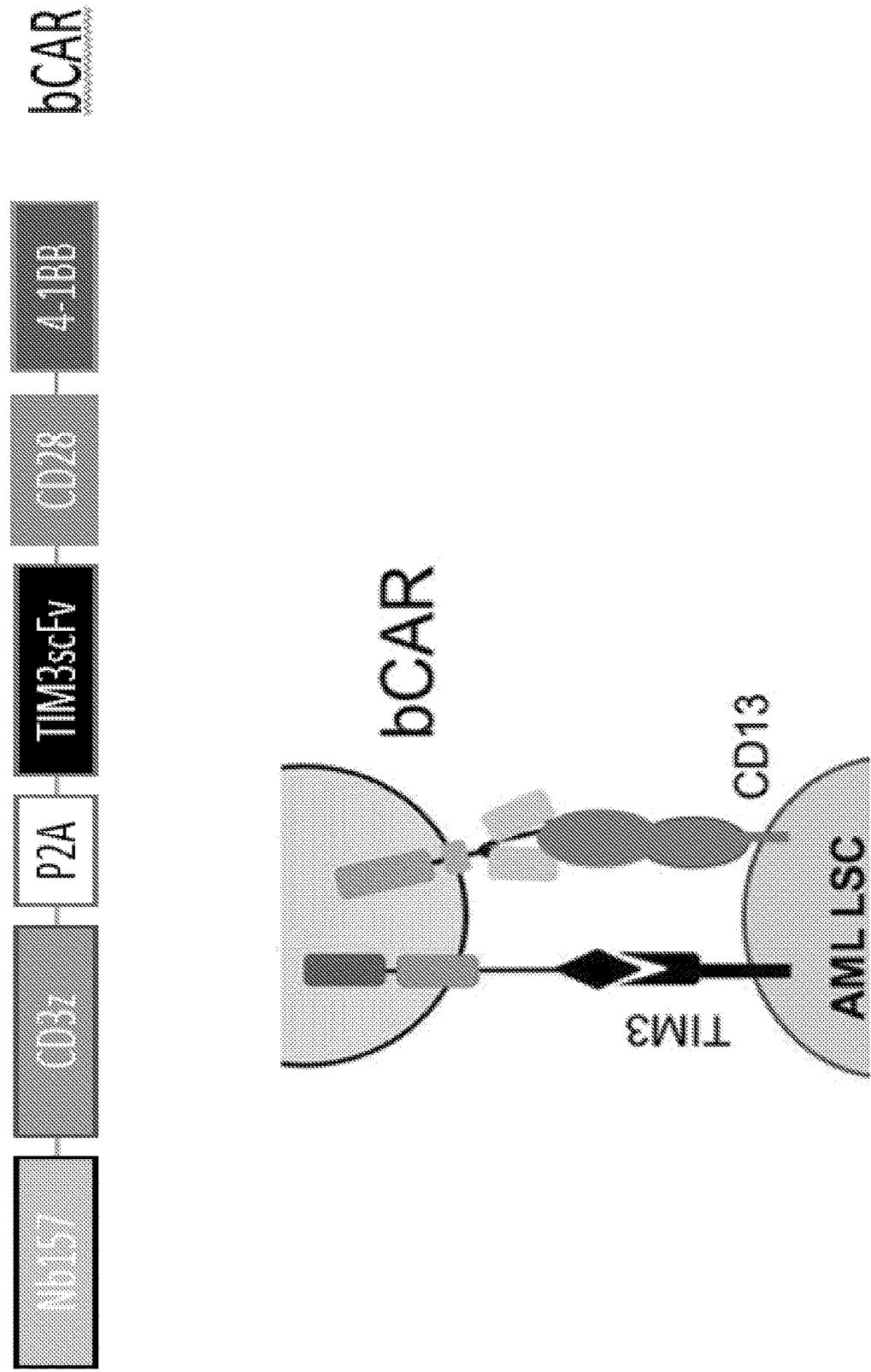


FIG. 2

FIG. 3A

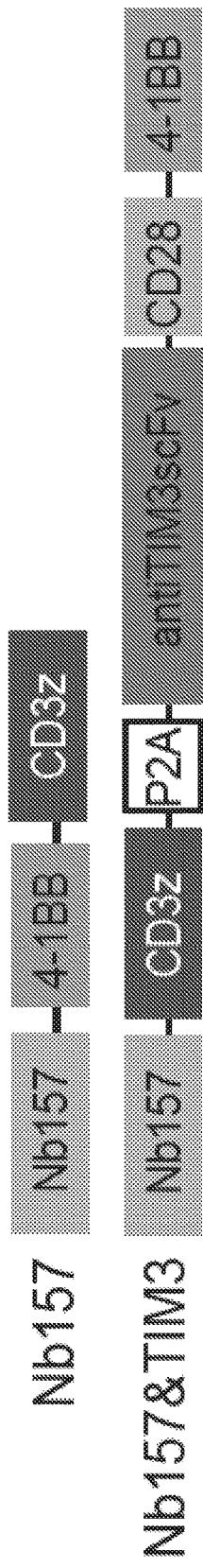
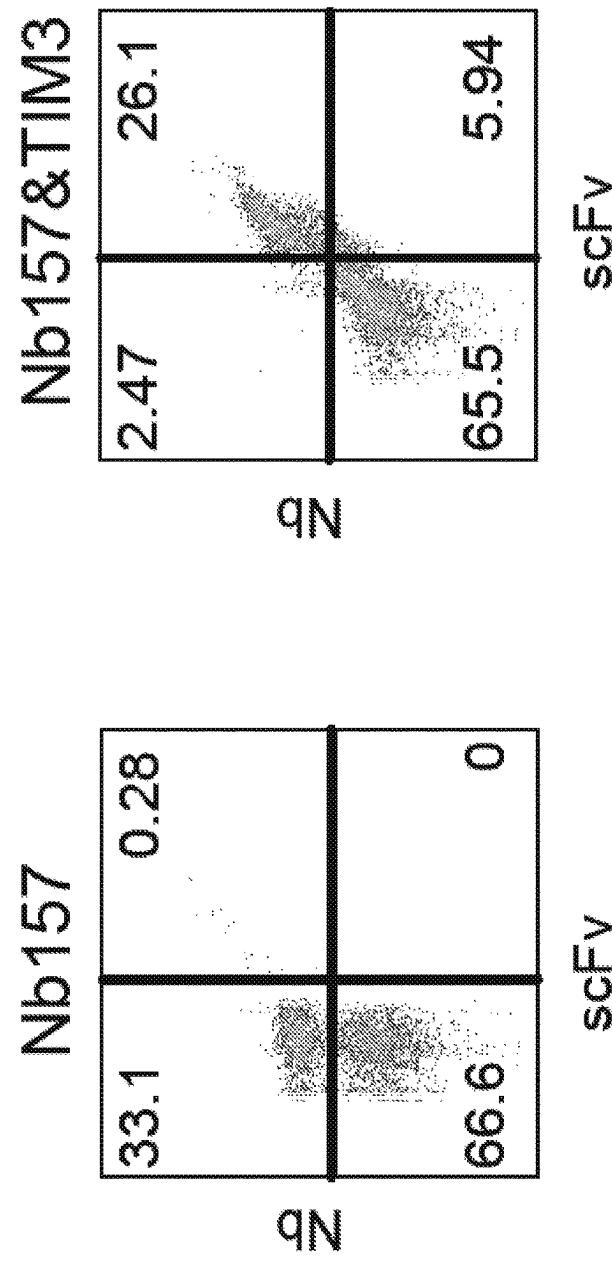


FIG. 3B



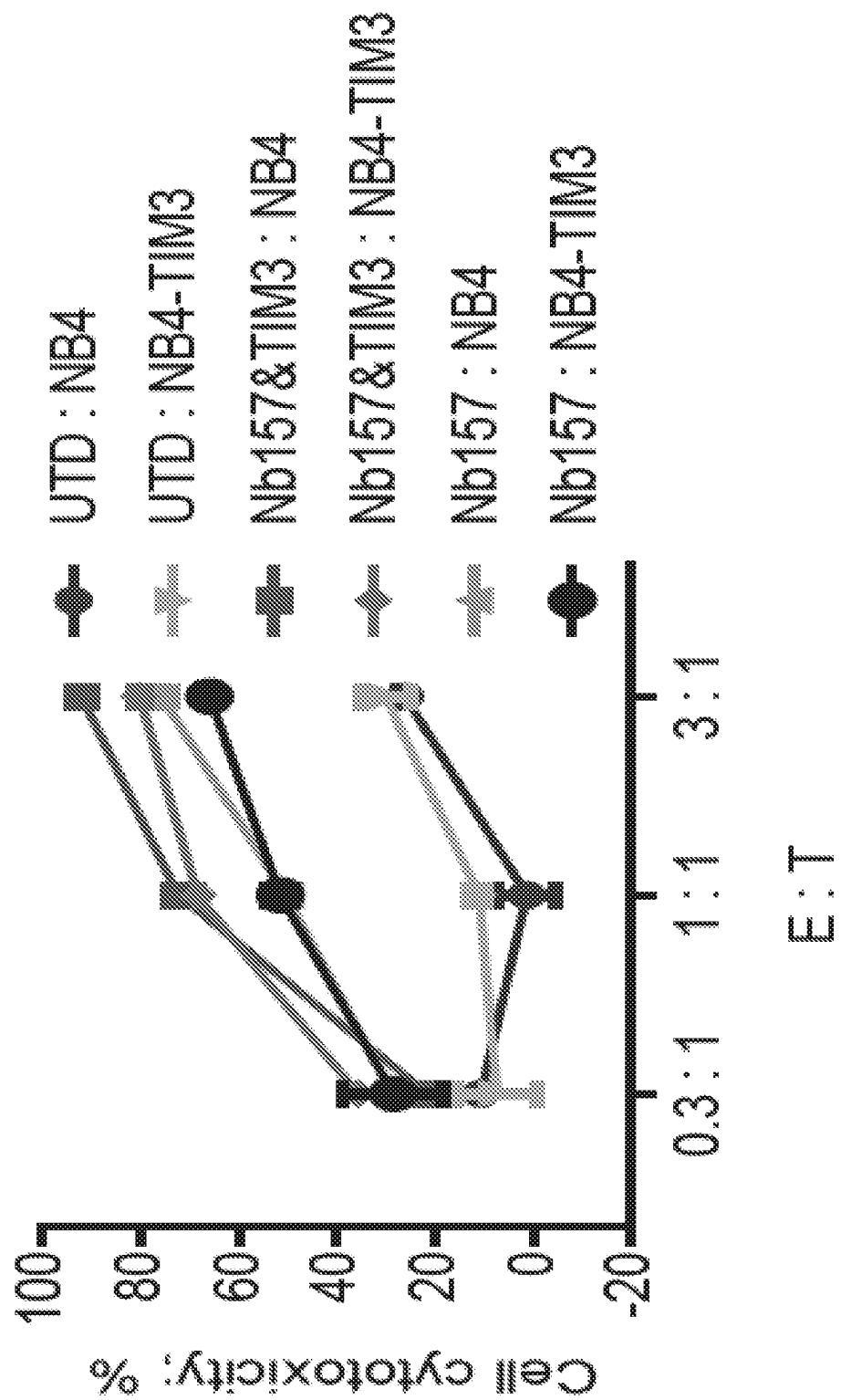


FIG. 3C

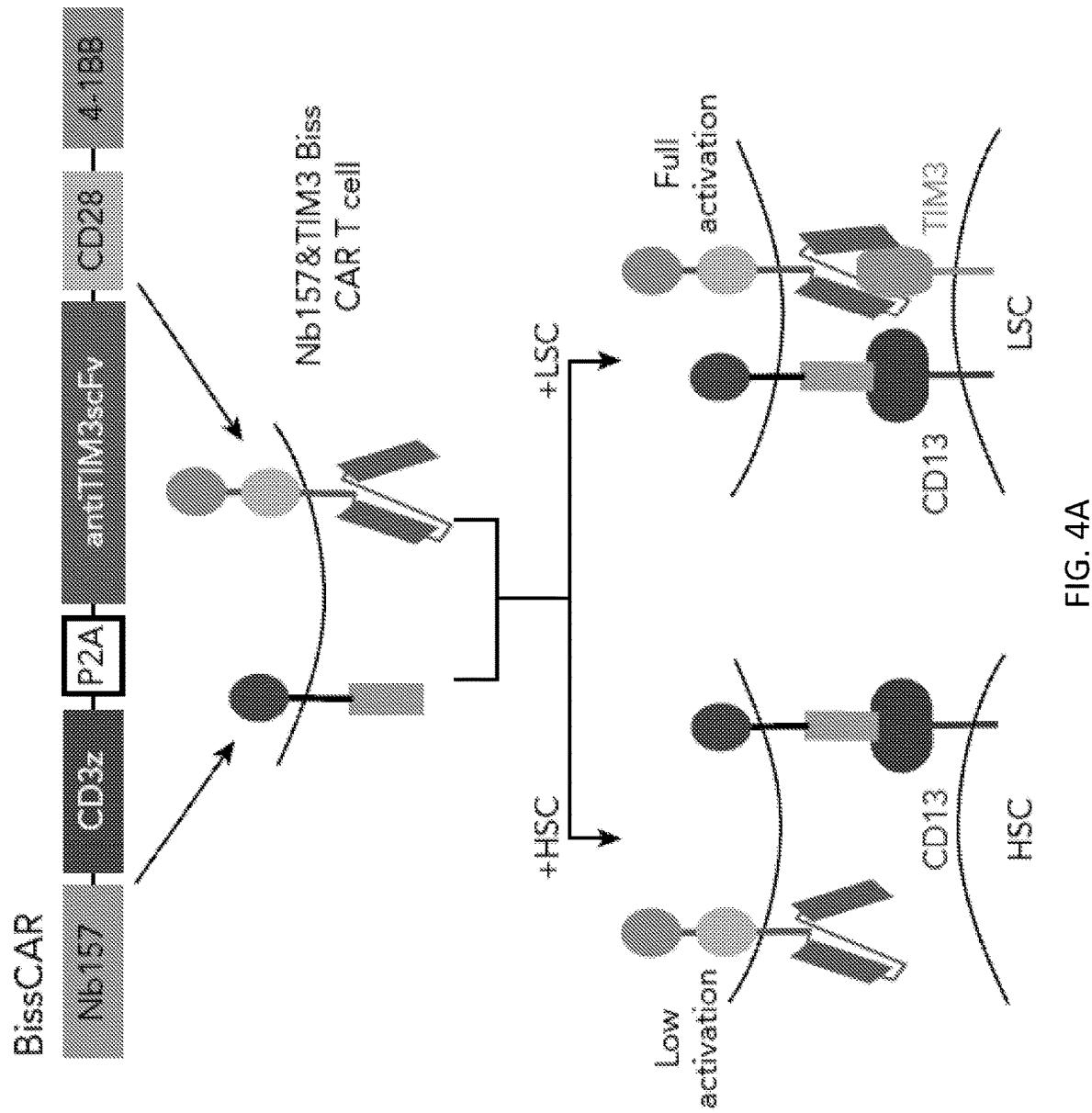
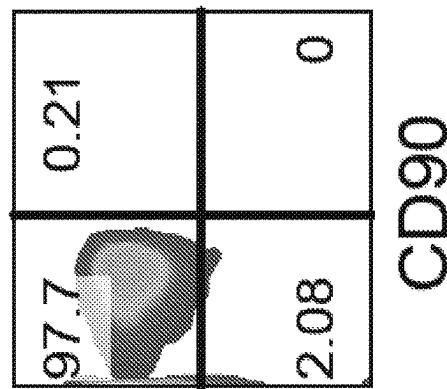


FIG. 4A

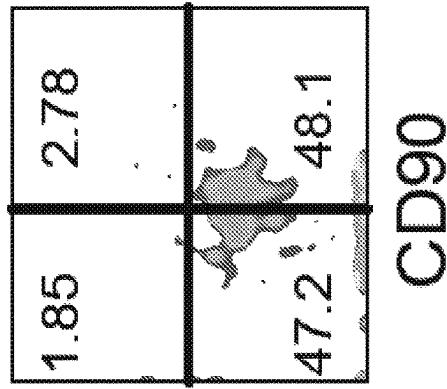
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TIM3

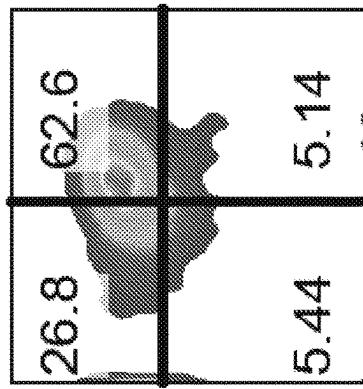
CD90

ND-BM



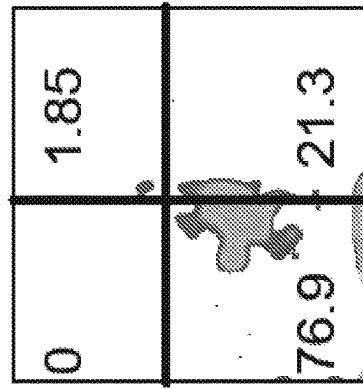
TIM3

CD90



TIM3

CD13



TIM3

CD13

FIG. 4B

FIG. 5A

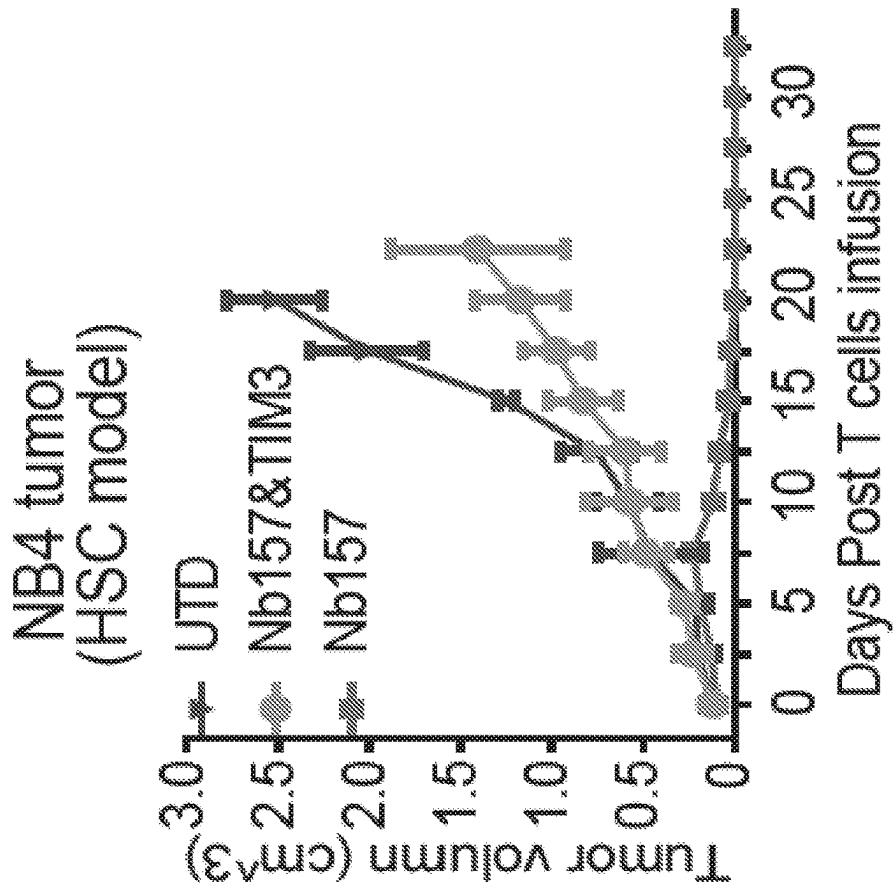
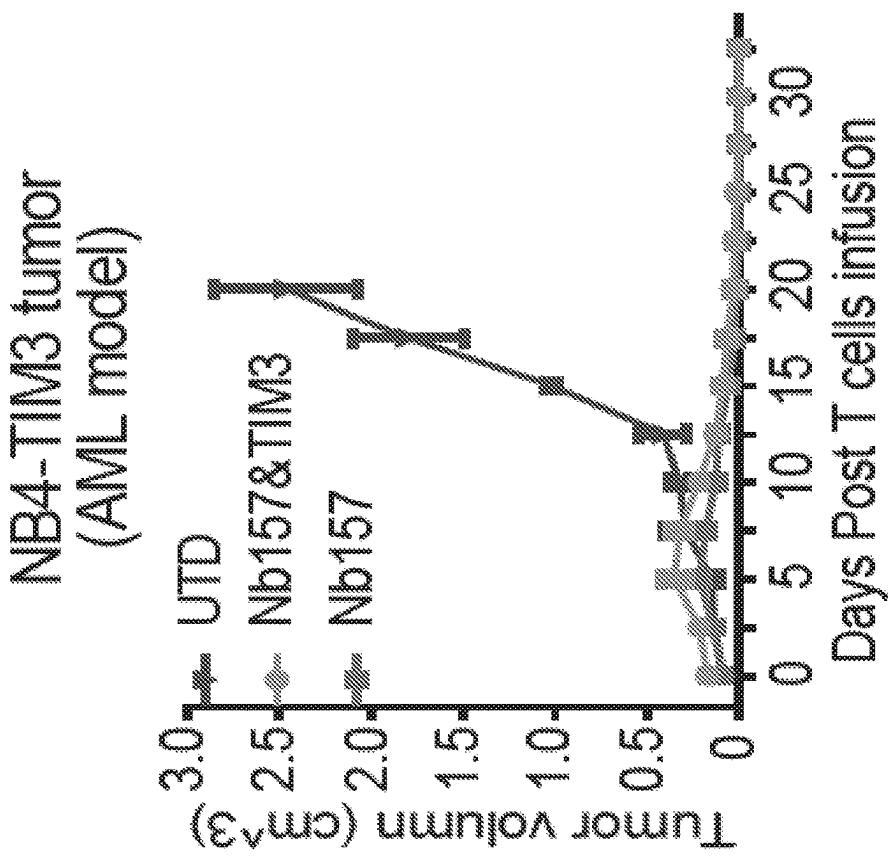


FIG. 5B



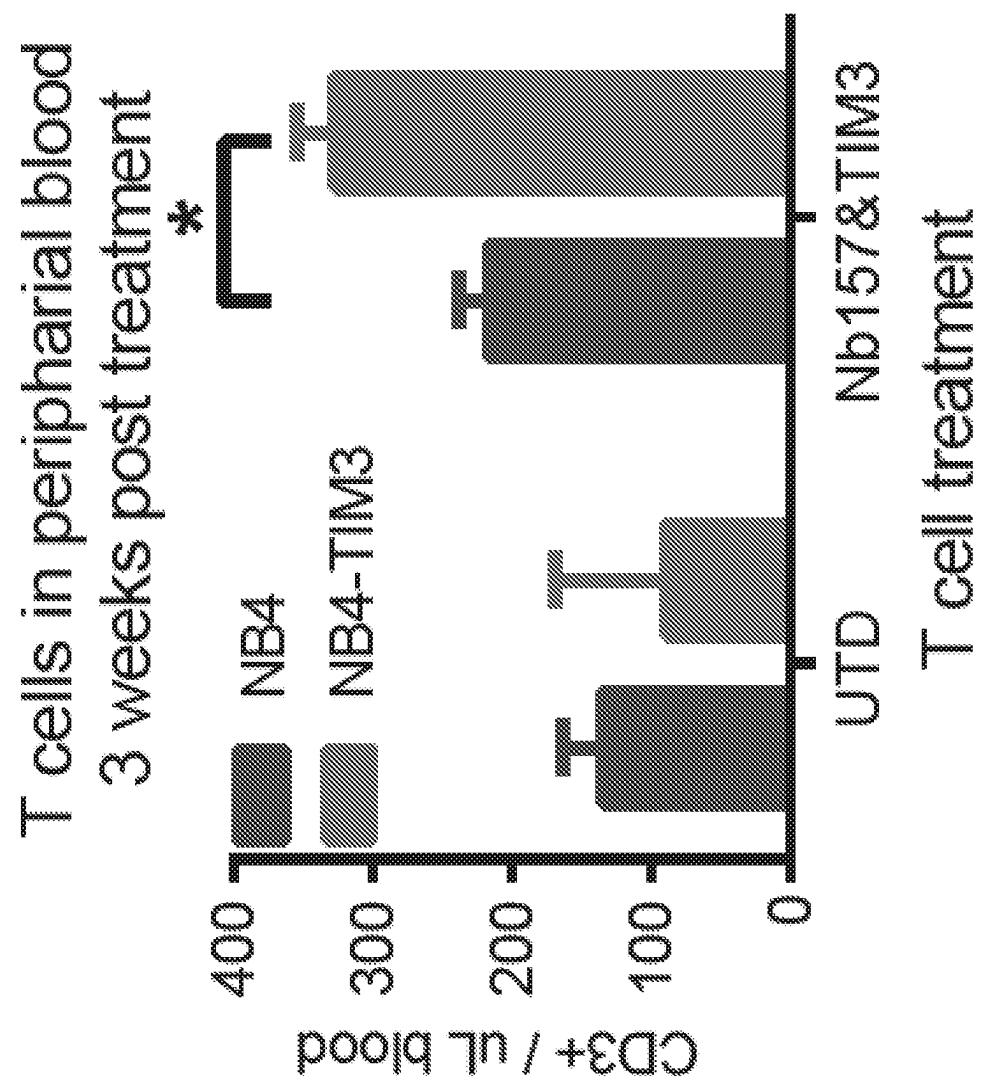


FIG. 6

FIG. 7A

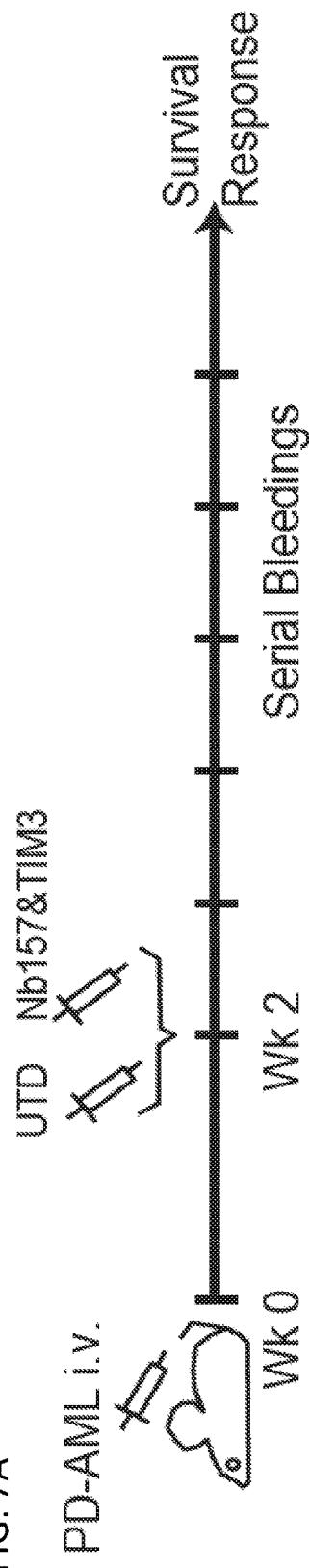


FIG. 7B

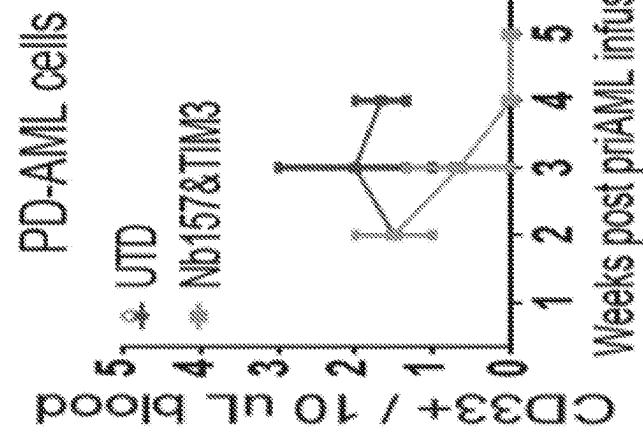


FIG. 7C

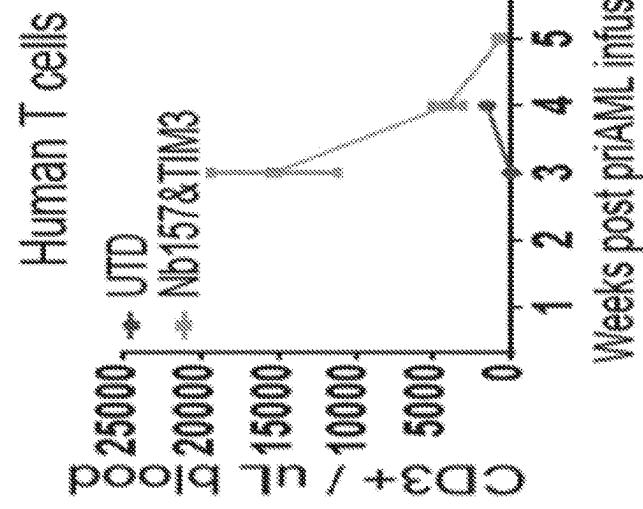
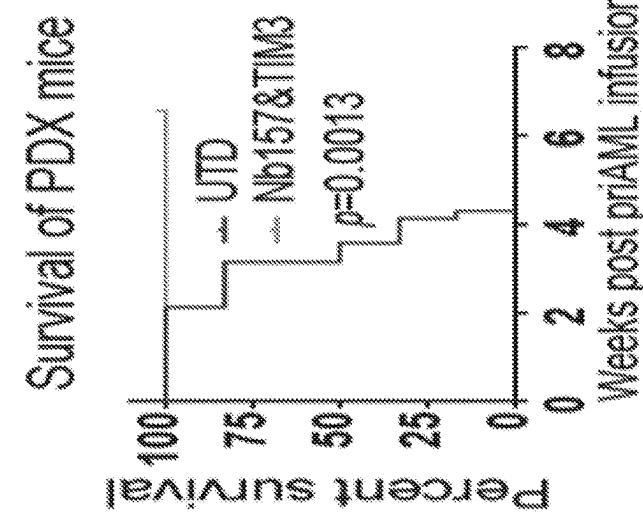
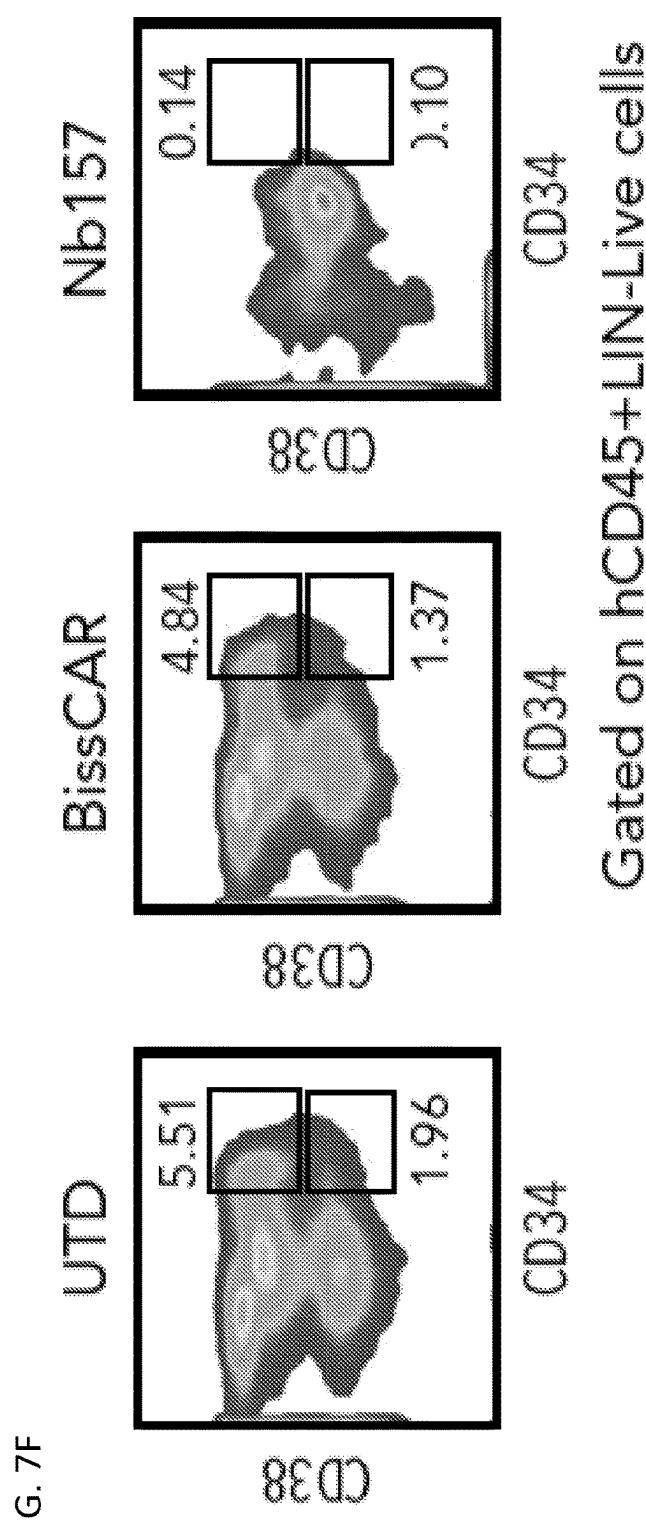
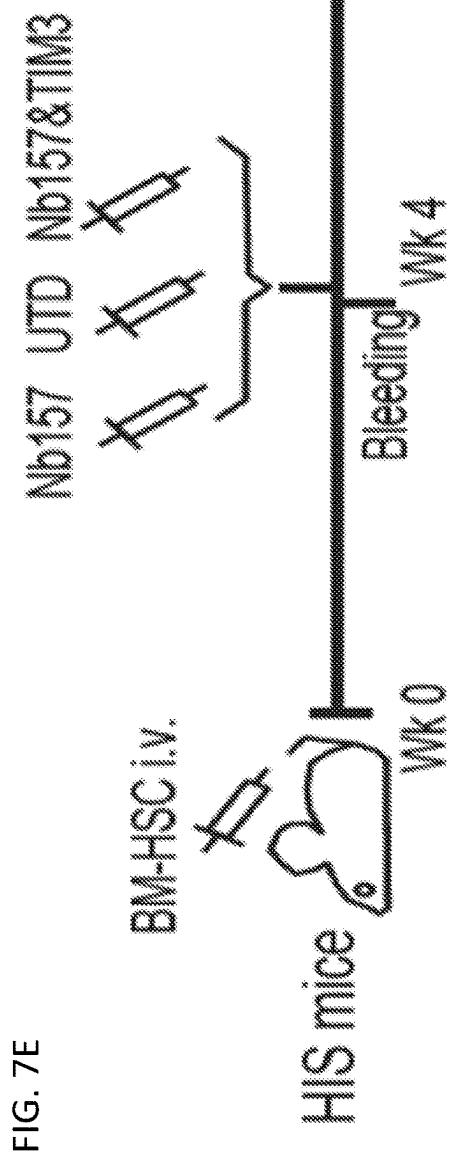


FIG. 7D





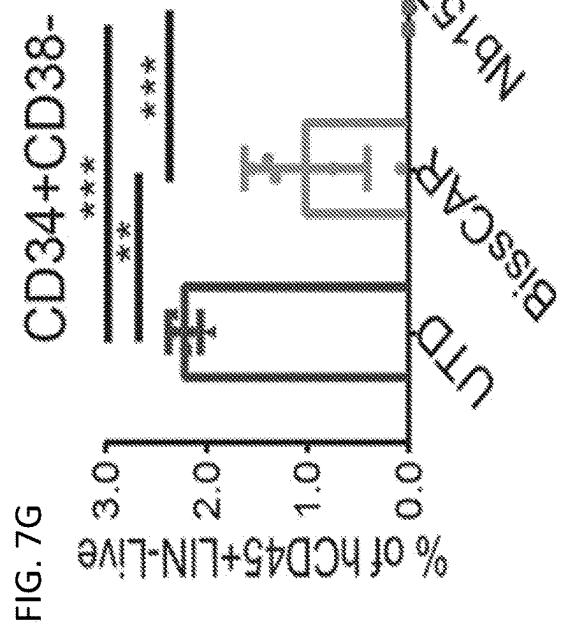


FIG. 7H

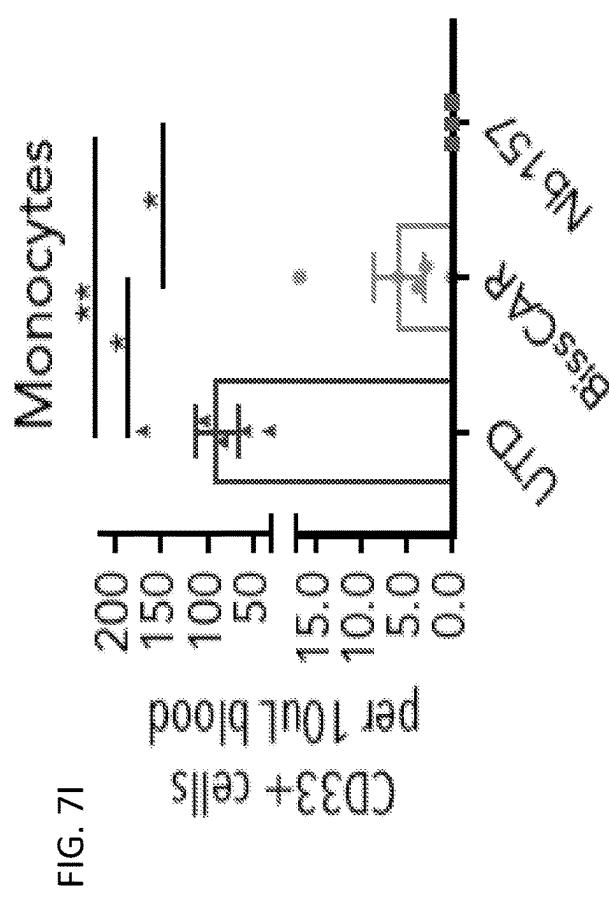
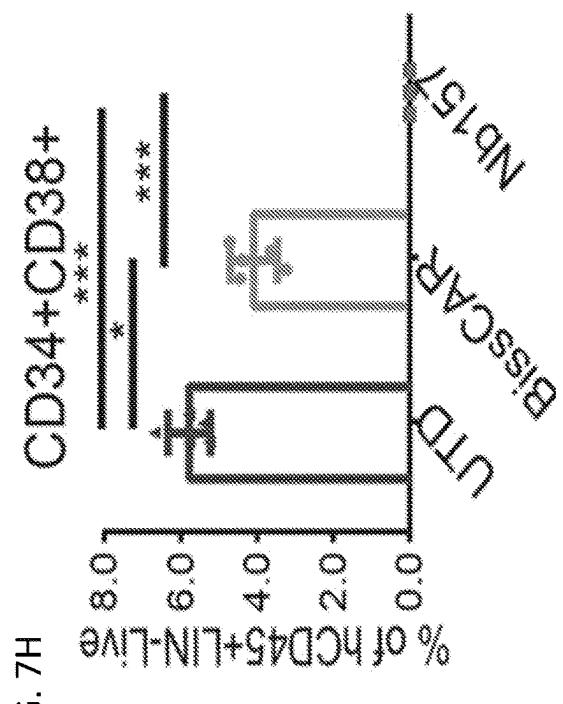


FIG. 7I

FIG. 8A

TIM3scFv    CD28    CD3z  
P2A

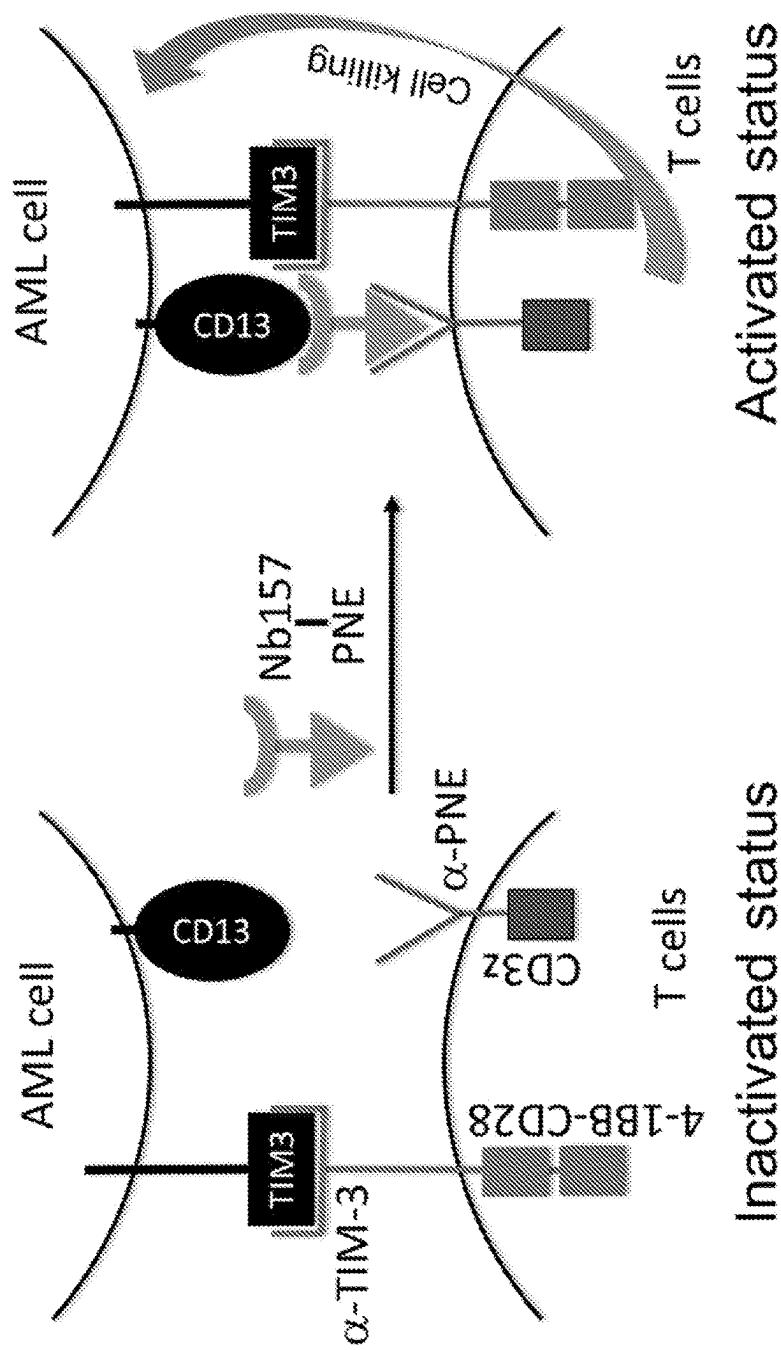


FIG. 8B

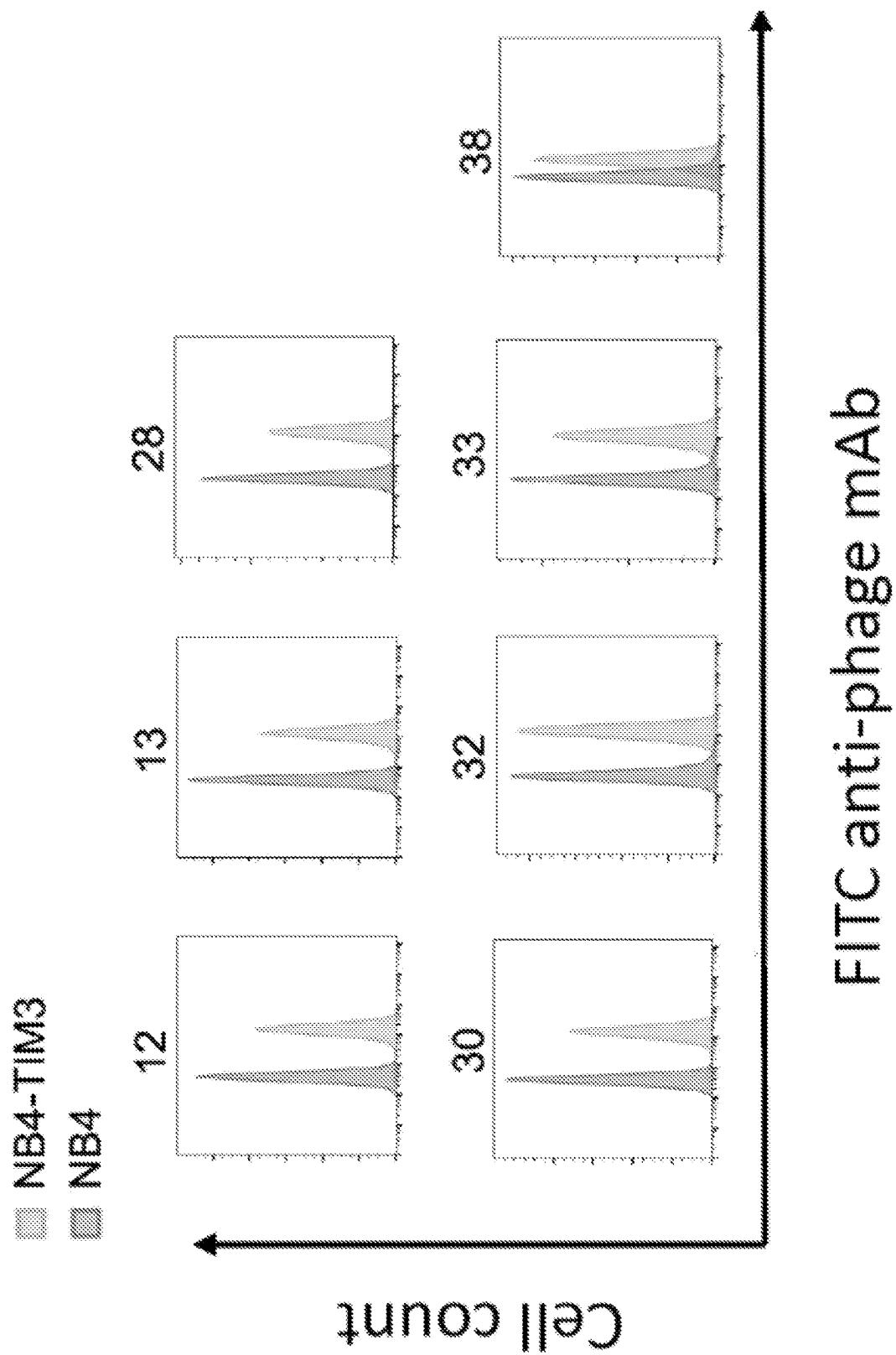


FIG. 9

FIG. 10A

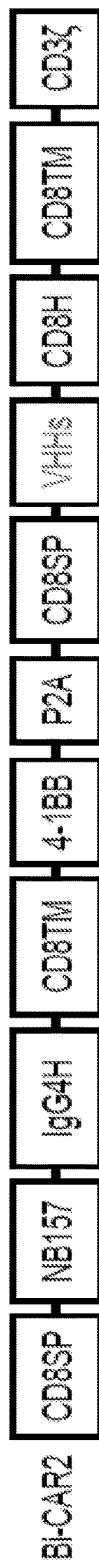
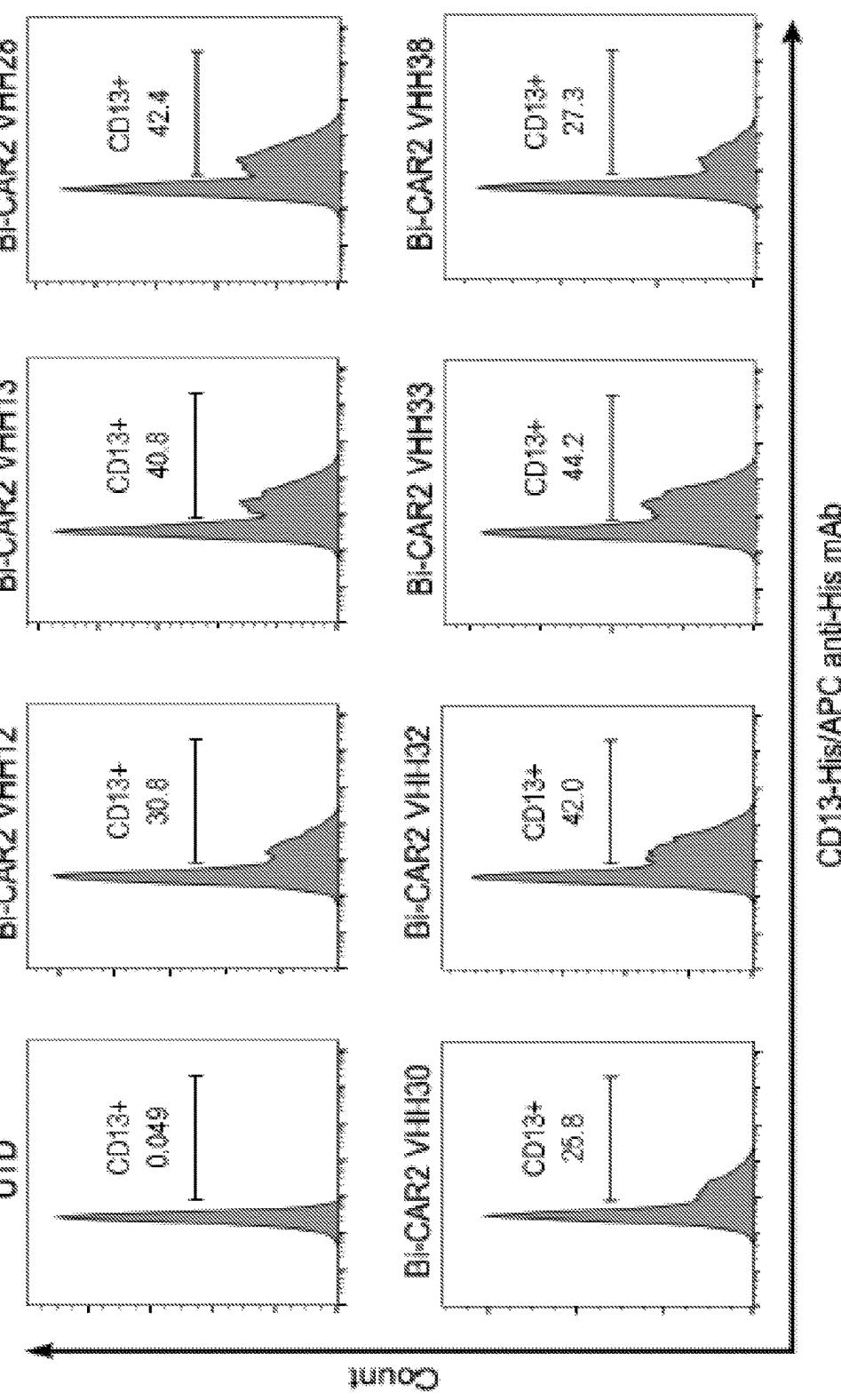


FIG. 10B



CD13-His/APC anti-His mAb

FIG. 10C

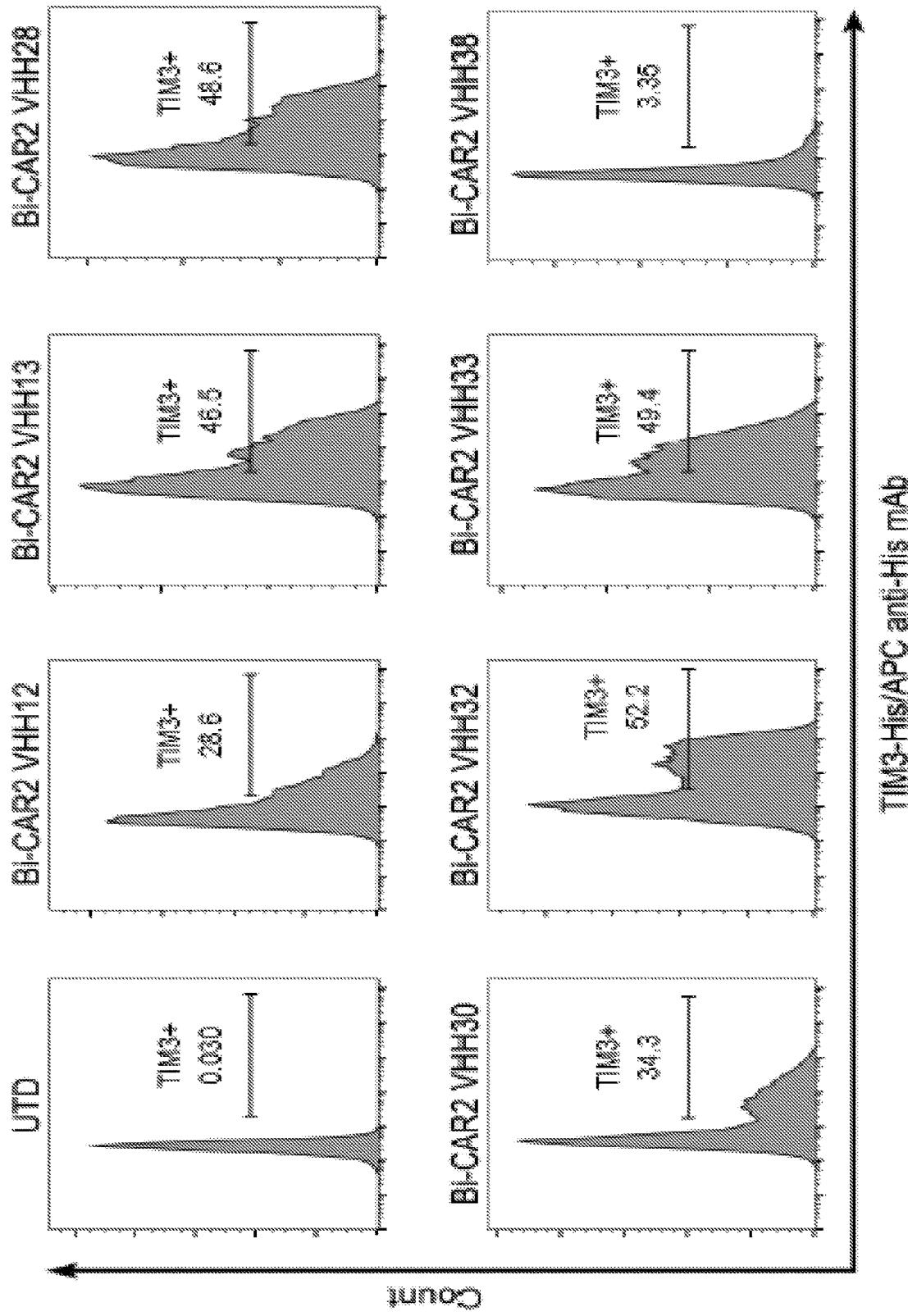


FIG. 11A

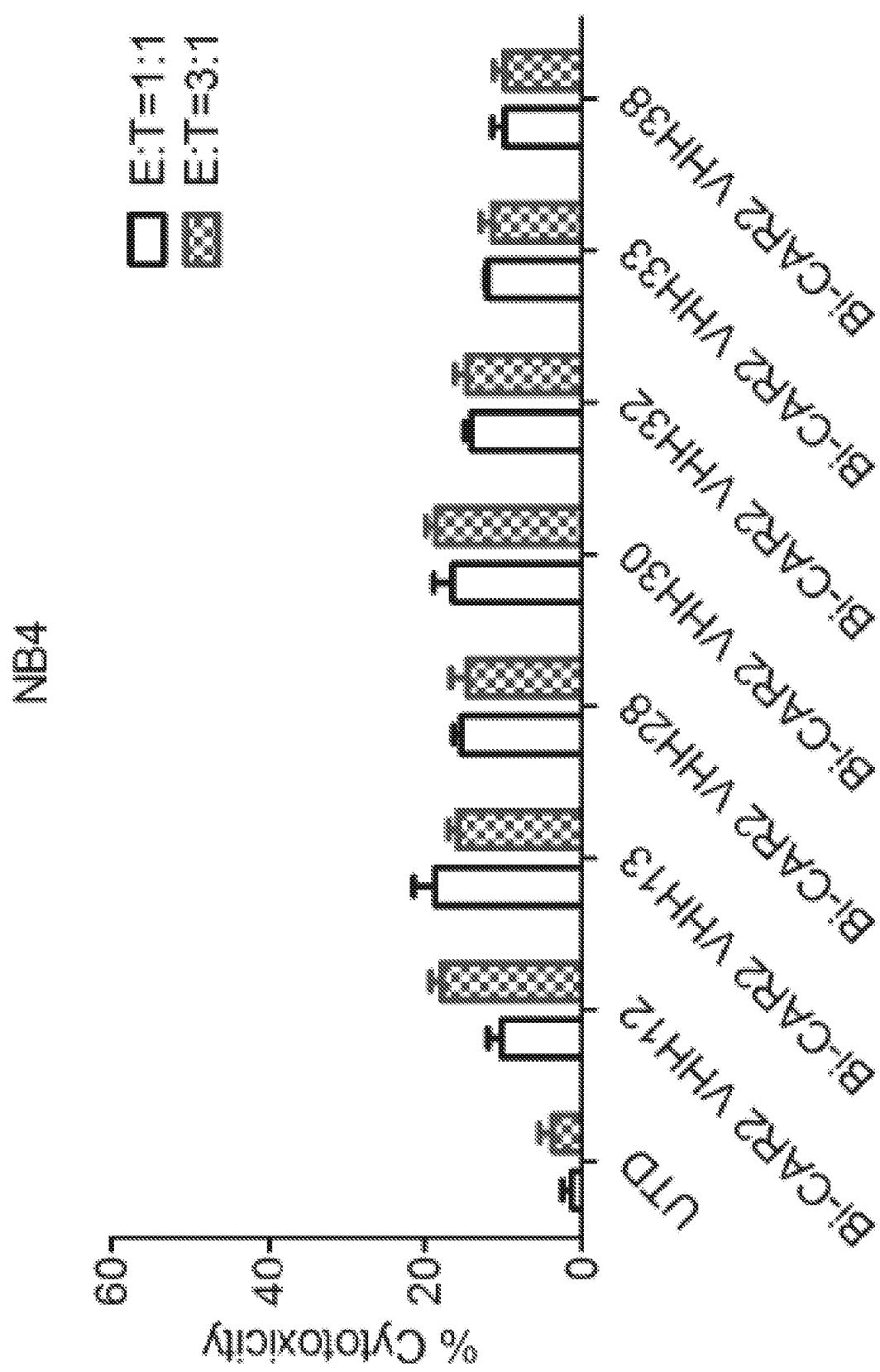
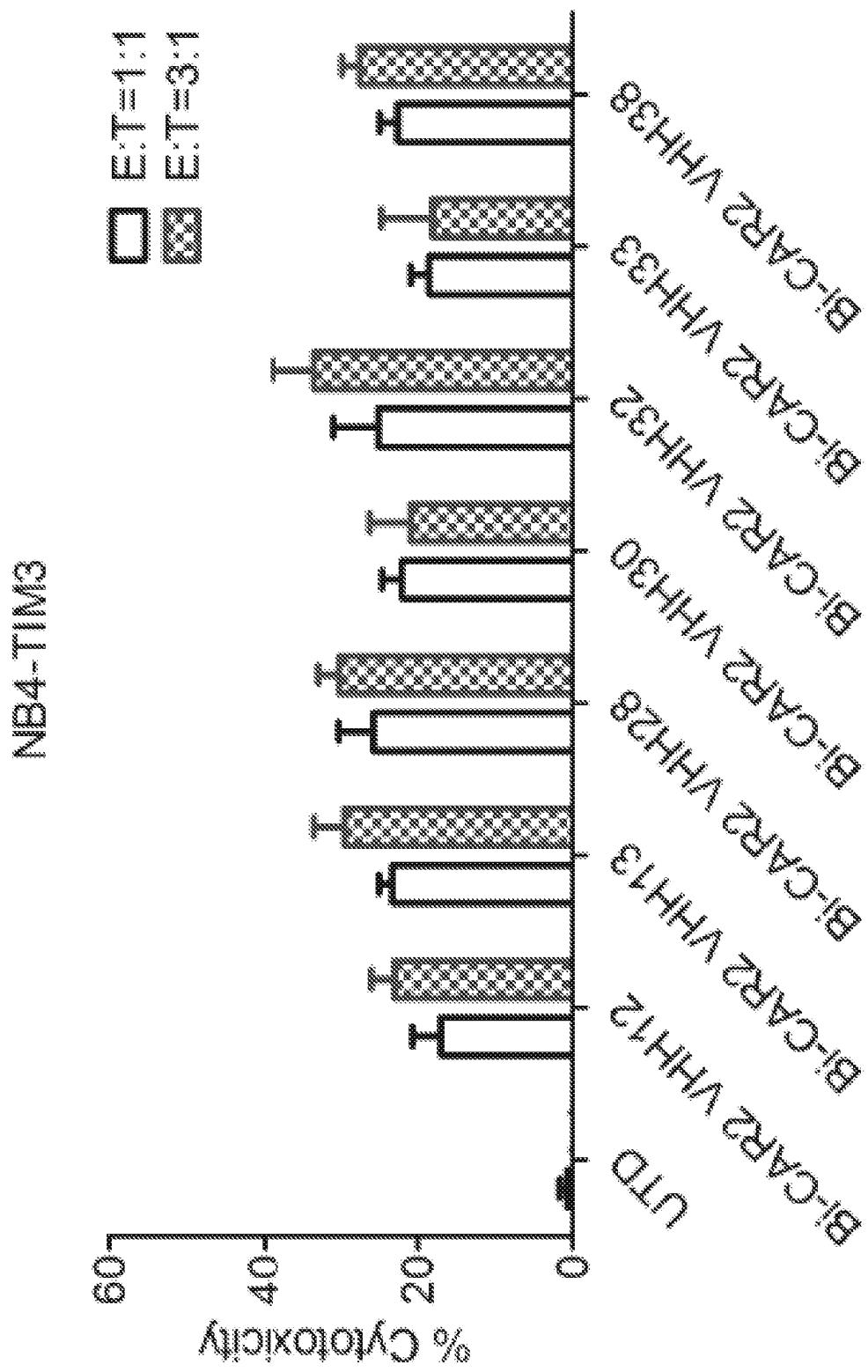


FIG. 11B



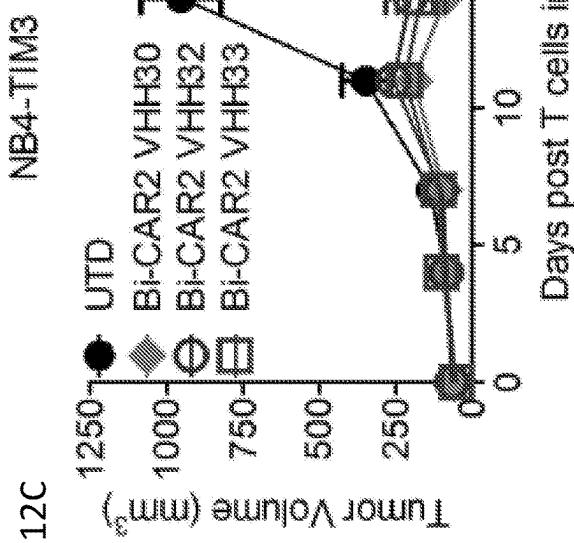
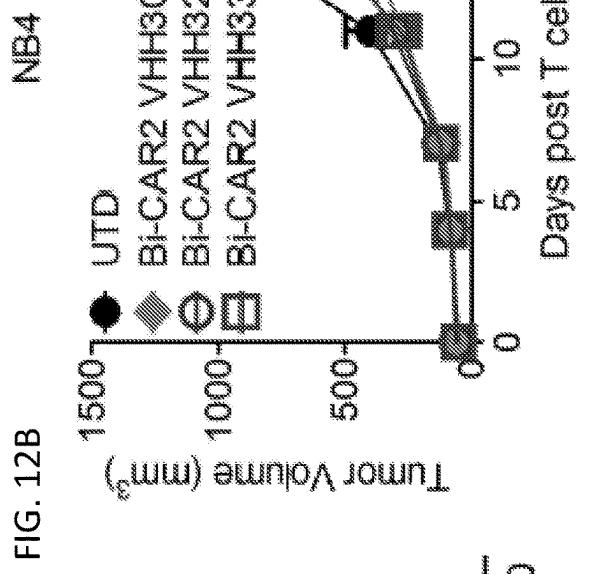
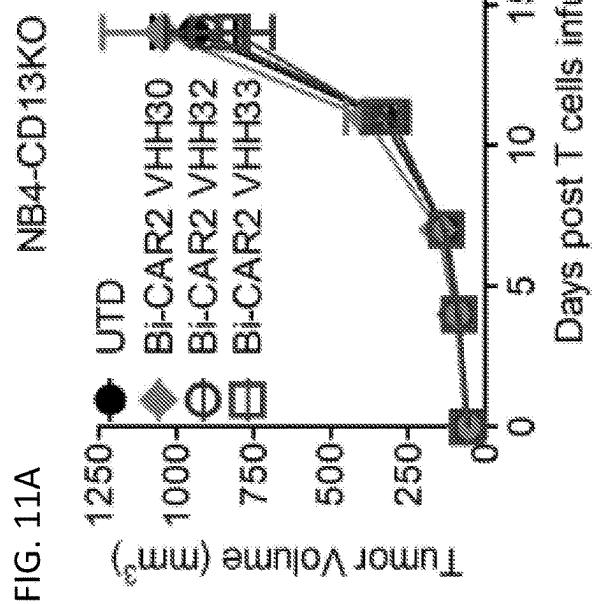


FIG. 12D T cells in peripheral blood

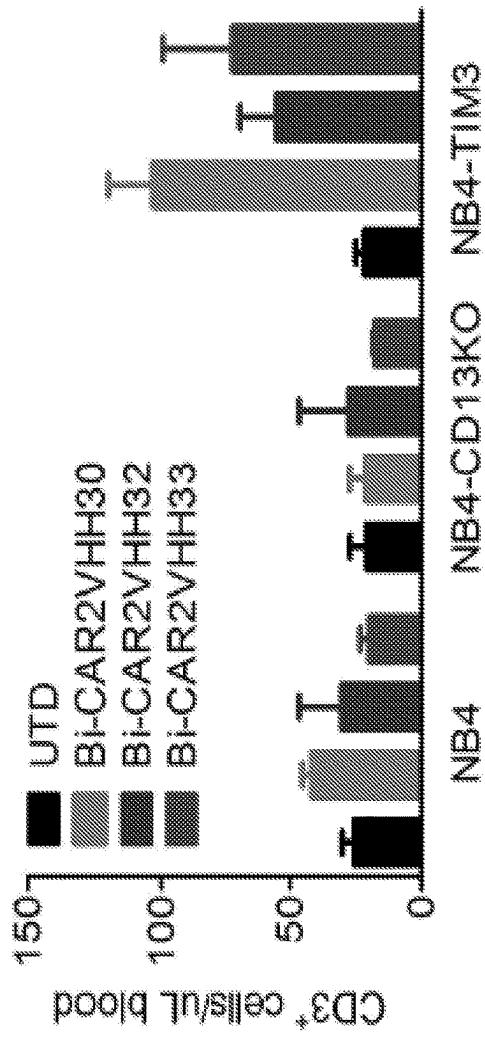
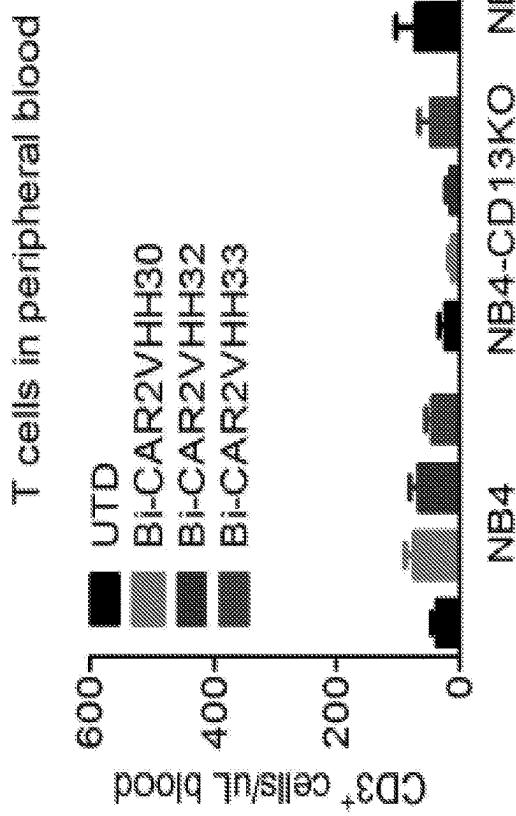


FIG. 12E



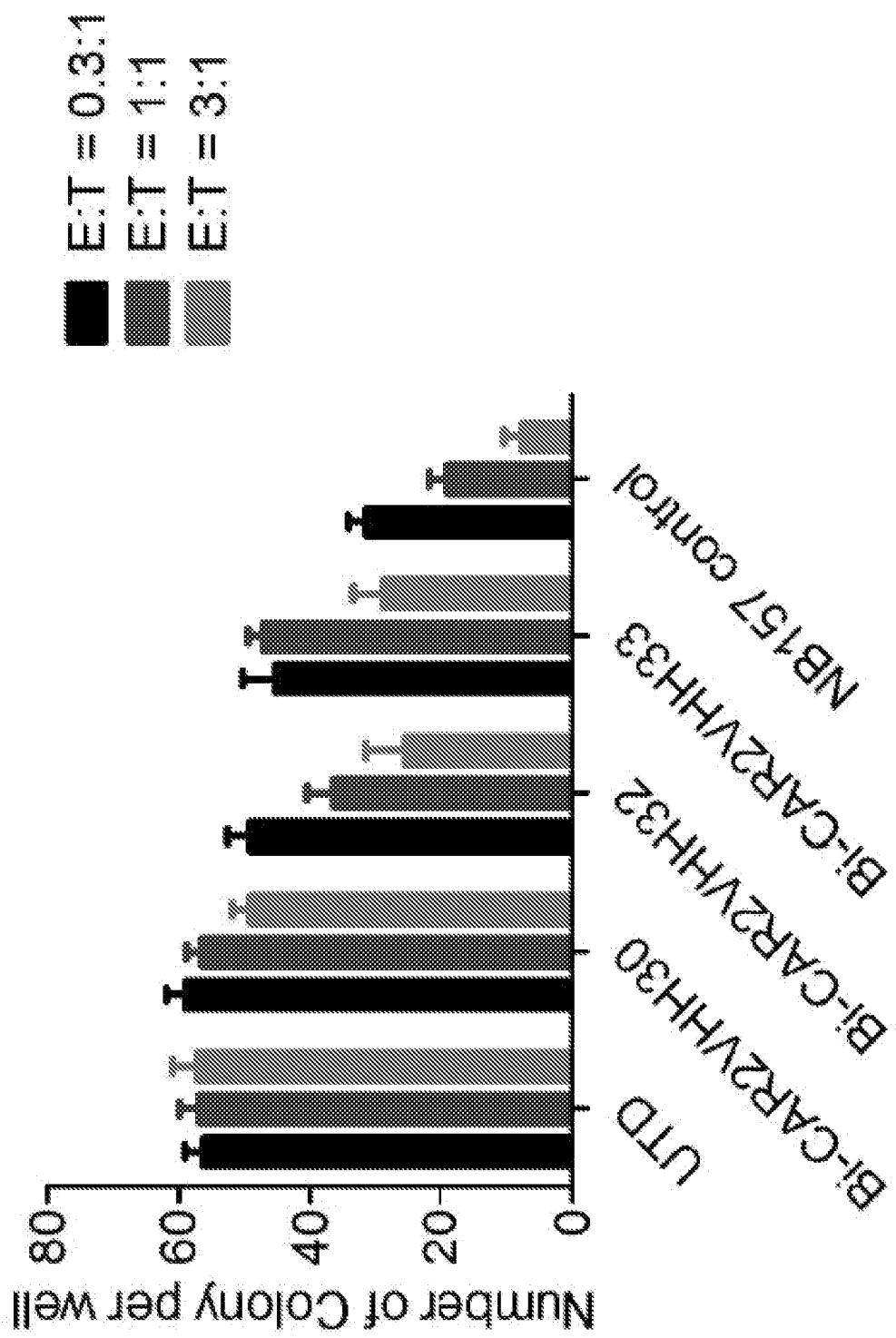


FIG. 13A

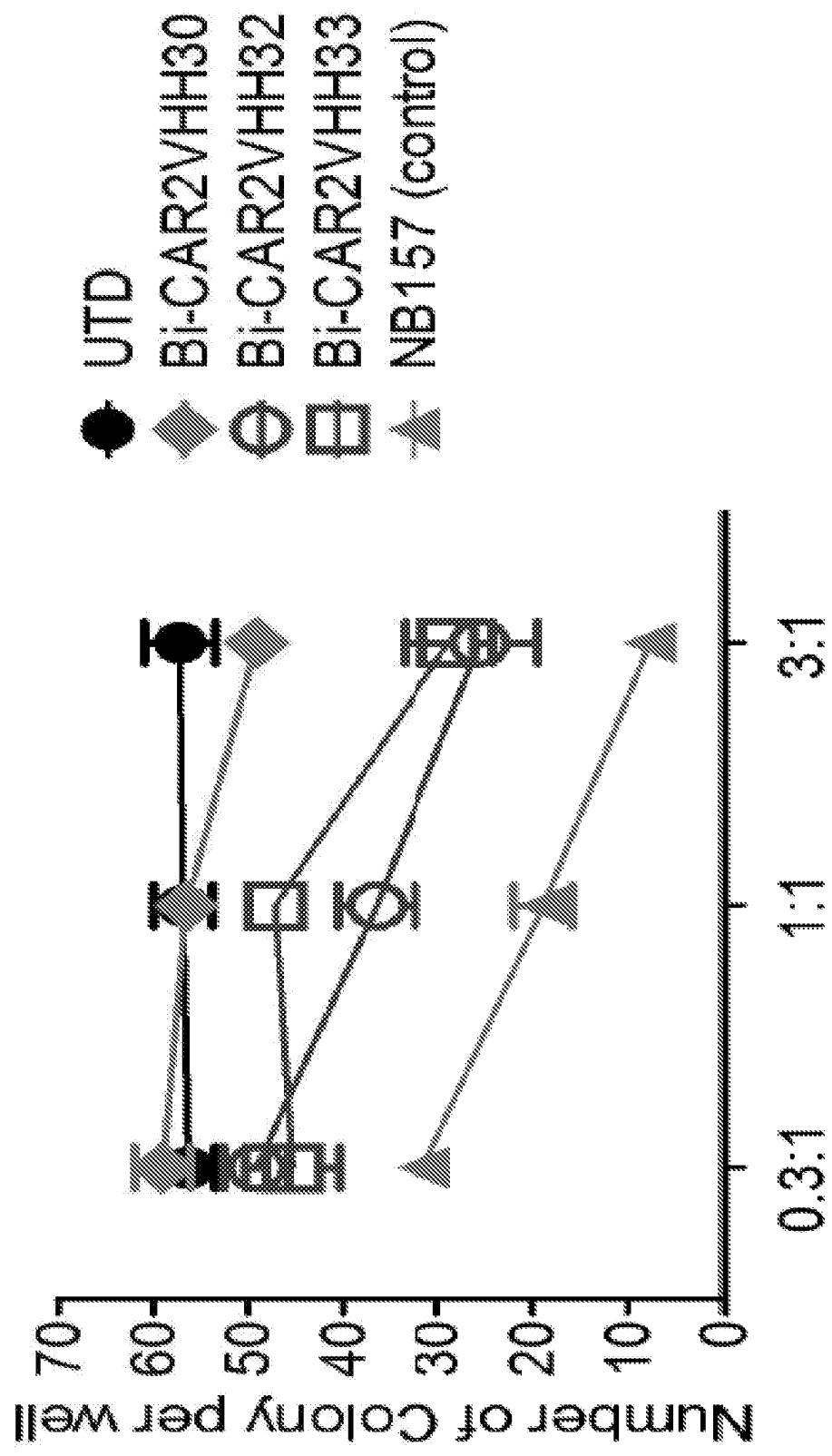


FIG. 13B

**1**

**COMPOSITIONS AND METHODS FOR  
TARGETING CD13 AND TIM-3 WITH CAR T  
CELLS TO TREAT ACUTE MYELOID  
LEUKEMIA**

**CROSS-REFERENCE TO RELATED  
APPLICATION**

The present application is a 35 U.S.C. § 371 national phase application from, and claims priority to, International Application No. PCT/US2020/055138, filed Oct. 9, 2020, and is entitled to priority under 35 U.S.C. § 119 (e) to U.S. Provisional Patent Application No. 62/913,915, filed Oct. 11, 2019, all of which are incorporated herein by reference in their entireties.

**REFERENCE TO AN ELECTRONIC SEQUENCE  
LISTING**

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Sep. 25, 2024 is named “046483-7259US1 (02850).txt and is 136,499 bytes in size.

**BACKGROUND OF THE INVENTION**

Acute myeloid leukemia (AML) is a major form of acute leukemia in elder adults. The treatment of AML has changed little in the past decades and the overall 5-year survival rate remains very poor in AML patients. AML relapse from chemotherapy is highly aggressive with poor prognosis. While adaptive cell therapy via chimeric antigen receptor (CAR)-expressing T cells is quite successful for treating acute and chronic lymphoblastic leukemia by targeting CD19, this approach has not yet been extensively explored for AML. There is an urgent need to develop potent antibodies against AML-specific surface targets to improve the therapy.

The CAR structure generally comprises an extracellular antigen recognition region and intracellular activation region. The recognition region typically comprises a single-chain variable fragment (scFv), composed of an antibody's heavy and light variable regions. The activation signal is transduced by the intracellular domains of the CD3 zeta, co-stimulatory 4-1BB and/or CD28. The co-stimulatory signals prolong survival and enhance cytotoxicity of CAR T cells to eliminate the cancer cells. CAR T cells targeting CD33, a cell surface lectin, and CD123, a subunit of IL-3 receptor, were tested for suppressing AML, but the approach was hindered by side effects.

A need exists for novel approaches for improving CAR T cell-mediated AML therapy. The present invention addresses this need.

**SUMMARY OF THE INVENTION**

As described herein, the present invention relates to compositions and methods for treating AML utilizing bispecific CARs (e.g. bispecific split CARs which bind CD13 and TIM-3 on AML cells.)

In one aspect, the invention provides a bispecific chimeric antigen receptor (CAR) comprising a first antigen binding domain capable of binding CD13, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain.

**2**

In certain embodiments, the first and/or second antigen binding domain is selected from the group consisting of an antibody, a nanobody, a Fab, or an scFv.

In certain embodiments, the second antigen binding domain comprises a nanobody, wherein the nanobody is Nb157.

In certain embodiments, the first antigen binding domain comprises the amino acid sequence set forth in SEQ ID NO: 1.

In certain embodiments, the second antigen binding domain comprises the amino acid sequence set forth in SEQ ID NO: 6.

In certain embodiments, the second antigen binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 20, 22, 24, 26, 28, 30, and 32. In certain embodiments, the second antigen binding domain is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 19, 21, 23, 25, 27, 29, and 31.

In certain embodiments, the transmembrane domain comprises CD28.

In certain embodiments, the first intracellular domain is selected from the group consisting of 4-1BB, CD28, and CD3 zeta. In certain embodiments, the second intracellular domain is selected from the group consisting of 4-1BB, CD28, and CD3 zeta.

In certain embodiments, the bispecific CAR further comprises a hinge domain selected from the group consisting of a CD8 hinge, an IgG3s hinge, and an IgG4m hinge.

In certain embodiments, the bispecific CAR is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs: 33-39.

In another aspect, the invention provides an inducible bispecific CAR comprising a first antigen binding domain capable of binding Peptide-Neo-Epitope (PNE), a first transmembrane domain, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain.

In certain embodiments, the first and/or second antigen binding domain is selected from the group consisting of an antibody, a Fab, or an scFv.

In certain embodiments, the second antigen binding domain comprises the amino acid sequence set forth in any one of SEQ ID NOs: 6, 20, 22, 24, 26, 28, 30, and 32.

In certain embodiments, the first and/or second transmembrane domain comprises CD28.

In certain embodiments, the first intracellular domain comprises CD3 zeta. In certain embodiments, the second intracellular domain comprises 4-1BB.

In certain embodiments, the CAR further comprises a hinge domain selected from the group consisting of a CD8 hinge, an IgG3s hinge, and an IgG4m hinge.

In another aspect, the invention provides a chimeric antigen receptor (CAR) comprising an antigen binding domain specific for TIM-3, a transmembrane domain, and an intracellular signaling domain.

In certain embodiments, the antigen binding domain is selected from the group consisting of an antibody, a nanobody, a Fab, or an scFv.

In certain embodiments, the antigen binding domain comprises the amino acid sequence set forth in any one of SEQ ID NOs: 6, 20, 22, 24, 26, 28, 30, and 32.

In certain embodiments, the CAR further comprises a hinge domain selected from the group consisting of a CD8 hinge, an IgG3s hinge, and an IgG4m hinge.

In certain embodiments, the transmembrane domain is selected from the group consisting of CD8, CD28, and ICOS.

In certain embodiments, the intracellular domain comprises 4-1BB and CD3 zeta.

In certain embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 13. In certain embodiments, the CAR is encoded by the nucleotide sequence of SEQ ID NO: 14.

In another aspect, the invention provides a modified T cell or precursor thereof, comprising any of the CARs or bispecific CARs contemplated herein.

In certain embodiments, the T cell is autologous.

In another aspect, the invention provides a nucleic acid encoding any of the CARs or bispecific CARs contemplated herein.

In another aspect, the invention provides a method for treating cancer in a subject in need thereof. The method comprises administering to the subject a modified T cell or precursor thereof comprising the any of the CARs or bispecific CARs contemplated herein. In certain embodiments, the cancer is acute myeloid leukemia (AML).

#### BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of specific embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings exemplary embodiments. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

FIGS. 1A-1D are series of images and graphs depicting an anti-TIM3 CAR that empowers T cells to specifically kill TIM-3 expressing AML cells. FIG. 1A illustrates confirmation of TIM-3 expression in NB4-TIM-3 cell lines by Western Blot. The star indicates the glycosylated TIM-3 protein. FIG. 1B shows flow cytometry analysis of NB4 and NB4-TIM3 cells by staining them with the antibodies targeting either CD13 and TIM3, indicating that NB4 cells only expressed CD13 and NB4-TIM3 cells expressed both CD13 and TIM3. FIG. 1C is a schematic diagram of an anti-TIM3 CAR. FIG. 1D illustrates cytotoxicity of the anti-TIM-3 CAR T against NB4-TIM-3 cells. NB4 or NB4-TIM-3 cells were incubated with anti-TIM-3-scFv-CAR T cells overnight. Untransduced (UTD) T cells were used as a negative control. Cytotoxicity was detected by flow cytometry analysis of the survival THP-1 cells. The data demonstrate that anti-TIM3 CAR T cells specifically kill TIM-3-expressing myeloid leukemia cells.

FIG. 2 illustrates a bispecific split CAR (BissCAR) that can specifically kill tumor cells expressing both TIM-3 and CD13.

FIGS. 3A-3C illustrate and compare a 2<sup>nd</sup> generation CAR targeting CD13 and a bispecific split CAR targeting both CD13 and TIM3. FIG. 3A is a schematic showing a 2nd generation CAR targeting CD13 (Nb157) and a bispecific split CAR (BissCAR) targeting both CD13 and TIM3 (Nb157 & TIM3). FIG. 3B illustrates cell surface expression of the respective CARs on the transduced CAR T cells. FIG. 3C illustrates each CARs ability to kill NB4 or NB4-TIM3 cells in vitro (FIG. 3C).

FIGS. 4A-4B illustrate combinatorial bispecific and split CARs targeting both CD13 and TIM-3. Bispecific and split CAR (BissCAR) T cells more potently kill leukemic stem cells (LSCs), which express both TIM3 and CD13, but spare

normal HSCs, which express CD13, but not TIM3. FIG. 4A is a schematic diagram of combinatorial bispecific and split CARs of the Nb157 (anti-CD13) and anti-TIM3 system. Nb157 linked with CD3z recognized CD13 on normal HSCs or LSCs. Anti-TIM3 linked with CD28 and 4-1BB recognized TIM3 only on LSCs. Such Biss CAR T cells can be fully activated only by LSCs but not by HSCs. FIG. 4B shows flow cytometry data showing the expression of TIM3, CD90, and CD13 on normal donor bone marrow cells (ND-BM) and patient-derived AML (PD-AML) cells, which were gated on CD45<sup>+</sup>Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup> subsets. Flow cytometry analysis showed that normal donor (ND) bone marrow CD34<sup>+</sup>/CD38<sup>-</sup>/CD90 cells, an HSC-enriched population, identifiable in the ND sample, but very low in AML patient derived leukemia sample (PD-AML) (FIG. 4B, top panels). In contrast, the TIM3/CD13 double positive cells from the CD34<sup>+</sup>/CD38<sup>-</sup> cell population were significantly increased in the PD-AML sample.

FIGS. 5A-5B show data from experiments wherein ten million NB4 or NB4-TIM3 cells were transplanted into NSG mice subcutaneously to form 100 mm<sup>3</sup> tumors. Three million Nb157&TIM3 combinational BissCAR T cells, conventional Nb157 CAR T cells, or UTD T cells were injected intravenously into NSG mice separately. The engraftment volume was monitored by measuring the length and width of the tumor every other day (n=4).

FIG. 6 illustrates the human T cells in peripheral blood of NSG mice bearing either NB4 or NB4-TIM3 tumors following treatment with the indicated control (UTD) or the indicated CAR T cells. Three weeks after mice with NB4 or NB4-TIM3 tumors were treated with the BissCAR (Nb157 & TIM3) T cells or UTD T cells, human T cell (CD3+) numbers in mouse peripheral blood were analyzed by flow cytometry and quantified using CountBright counting beads (n=3). \*P<0.05, Student t test.

FIGS. 7A-7H illustrate BissCAR T cells targeting CD13 and TIM3 eradicate AML PDXs, but with reduced toxicity to human HSCs in vivo. FIG. 7A is a schematic diagram of an AML PDX mice treated with control or BissCAR T cells. Twenty million patient derived AML cells were injected into each NSG mouse, followed by injection of 5 million BissCAR T cells or UTD T cells, 2 weeks later. Human peripheral blood CD3<sup>+</sup> cells were analyzed by serial bleeding weekly. FIGS. 7B-7C depict patient-derived AML cells or T cells in mouse peripheral blood were monitored weekly by staining with anti-human CD33 or anti-human CD3 antibodies. FIG. 7D shows mice survival was monitored and recorded (n=6 per group). FIG. 7E is a schematic diagram of HIS mice for evaluation of human HSC toxicity. A total of 1.5 million normal donor bone marrow (BM) CD34<sup>+</sup> cells were injected into each NSG mouse. Four weeks later, 3 million Nb157 anti-TIM3 BissCAR T cells, or conventional Nb157 CARs, or UTD T cells were injected intravenously, followed by flow cytometry analysis of peripheral blood and bone marrow (n=5 per group for Biss CAR and UTD cells; n=3 per group of Nb157 cells). FIG. 7F shows bone marrow of HIS mice, which were treated with T cells for 3 weeks, was analyzed by flow cytometry after staining with CD45/Lin/CD34/CD38/7-AAD. Representative fluorescence-activated cell sorting plots were used to identify HSC (CD34<sup>+</sup> CD38<sup>+</sup>) and myeloid progenitors (CD34<sup>+</sup>CD38<sup>+</sup>). FIG. 7G shows HSCs (CD45<sup>+</sup>Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>) in the bone marrow of HIS mice were analyzed by flow cytometry 3 weeks after the initial treatment. FIG. 7H shows myeloid progenitors (CD45<sup>+</sup>Lin CD34<sup>+</sup>CD38<sup>-</sup>) in the bone marrow of HIS mice were analyzed by flow cytometry 3 weeks after the initial treatment. FIG. 7I shows monocytes (human CD45<sup>+</sup>

CD33<sup>+</sup>) from peripheral blood of HIS mice were analyzed by flow cytometry 3 weeks after the initial treatment; cell number and blood volume were quantified using Count-Bright counting beads. In FIG. 7G-7I, n=5 per group for BissCAR T cells and UTD T cells, n=3 per group for Nb157 T cells. \*P<. 05, \*\*P<01, \*\*\*P<. 001, Student t test.

FIGS. 8A-8B illustrate a TIM-3& PNE inducible bispecific CAR.

FIG. 9 illustrates identification of unique individual anti-TIM3 VHVs analyzed by flow cytometry. Isolation of individual VHH phage clones from library following TIM3-negative and positive NB4 screening.

FIGS. 10A-10B illustrate bispecific CAR comprising anti-TIM3 VHVs. FIG. 10A is a schematic illustrating construction of bispecific CARs using newly generated anti-TIM3 VHVs. FIGS. 10B-10C illustrate expression of the bispecific CARs on primary human T cells.

FIGS. 11A-11B illustrate the killing effects of bispecific CARs against NB4 (FIG. 11A) and NB4-TIM3 (FIG. 11B) cells.

FIGS. 12A-12E illustrate the specificity of the various bispecific CARTs in suppressing tumors in vivo. The anti-tumor effects of bispecific CAR T cells against NB4-CD13KO (FIG. 12A), NB4 (FIG. 12B) and NB4-TIM3 (FIG. 12C) tumors were evaluated. Various NB4 cells were injected subcutaneously into each flank of a NSG mice (n=3), and the indicated CARTs ( $5 \times 10^6$ ) were injected into each mouse via tail vein at 7 days after tumor cell injection. The number of human T cells in peripheral blood of mice bearing NB4-CD13KO, NB4 and NB4-TIM3 tumors was measured by flow cytometry 7 days (FIG. 12D) and 14 days (FIG. 12E) after T cell infusion.

FIGS. 13A-13B illustrate data evaluating the potential toxicity of the bispecific CAR T cells against human bone marrow CD34+ cells. BM CD34+ cells (5000 per well) were co-cultured with the indicated T cells (0.3:1, 1:1, 3:1) for 4 hours. Cells were transferred into 12-well plates and cultured in MethoCult™ H4435 Enriched medium. Two weeks later, the number of clones was measured. FIG. 13A shows the number of colonies from control (UTD) or the bispecific CART treated plates. FIG. 13B is a linear graph comparing the dose-dependent effect of the CARTs based on colony number.

## DETAILED DESCRIPTION

### 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 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.

It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of  $\pm 20\%$  or  $\pm 10\%$ , more

preferably  $\pm 5\%$ , even more preferably  $\pm 1\%$ , and still more preferably  $\pm 0.1\%$  from the specified value, as such variations are appropriate to perform the disclosed methods.

"Activation," as used herein, refers to the state of a T cell that has been sufficiently 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.

As used herein, to "alleviate" a disease means reducing the severity of one or more symptoms of the disease.

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

"Alloantigen" refers to an antigen present only in some individuals of a species and capable of inducing the production of an alloantibody by individuals which lack it.

The term "antibody," as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)<sub>2</sub>, as well as single chain antibodies (scFv) 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')<sub>2</sub>, 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.  $\alpha$  and  $\beta$  light chains refer to the two major antibody light chain isotypes.

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 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.

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 any material derived from a different animal of the same species. The term "chimeric antigen receptor" or "CAR," as used herein, refers to an artificial T cell receptor that is engineered to be expressed on an immune effector cell and specifically bind an antigen. CARs may be used as a therapy with adoptive cell transfer. T cells are removed from a patient and modified so that they express the receptors specific to a particular form of antigen. In some embodiments, the CAR has specificity to a selected target, for example a tumor antigen. CARs may also comprise an intracellular activation domain, a transmembrane domain and an extracellular domain comprising an antigen binding region.

"Co-stimulatory ligand," as the term is used herein, includes a molecule on an antigen presenting cell (e.g., an aAPC, dendritic cell, B cell, and the like) that specifically binds a cognate co-stimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary signal provided by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, mediates a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A co-stimulatory ligand can include, but is not limited to, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, HVEM, lymphotoxin beta receptor, 3/TR6, ILT3, ILT4, HVEM, an agonist or antibody that binds Toll ligand receptor and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also encompasses, *inter alia*, an antibody that specifically binds with a co-stimulatory molecule present on a T cell, such as, but not limited to, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a T cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the T cell, such as, but not limited to, proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and a Toll ligand receptor.

A "co-stimulatory signal", as used herein, refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation and/or upregulation or downregulation of key molecules.

A "disease" is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal's health continues to deteriorate. In contrast, a "disorder" in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal's state of health.

The term "downregulation" as used herein refers to the decrease or elimination of gene expression of one or more genes.

"Effective amount" or "therapeutically effective amount" are used interchangeably herein, and refer to an amount of

a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result or provides a therapeutic or prophylactic benefit. Such results may include, but are not limited to an amount that when administered to a mammal, causes a detectable level of immune suppression or tolerance compared to the immune response detected in the absence of the composition of the invention. The immune response can be readily assessed by a plethora of art-recognized methods. The skilled artisan would understand that the amount of the composition administered herein varies and can be readily determined based on a number of factors such as the disease or condition being treated, the age and health and physical condition of the mammal being treated, the severity of the disease, the particular compound being administered, and the like.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

As used herein "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

The term "epitope" as used herein is defined as a small chemical molecule on an antigen that can elicit an immune response, inducing B and/or T cell responses. An antigen can have one or more epitopes. Most antigens have many epitopes; i.e., they are multivalent. In general, an epitope is roughly about 10 amino acids and/or sugars in size. Preferably, the epitope is about 4-18 amino acids, more preferably about 5-16 amino acids, and even more most preferably 6-14 amino acids, more preferably about 7-12, and most preferably about 8-10 amino acids. One skilled in the art understands that generally the overall three-dimensional structure, rather than the specific linear sequence of the molecule, is the main criterion of antigenic specificity and therefore distinguishes one epitope from another. Based on the present disclosure, a peptide of the present invention can be an epitope.

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

The term "expand" as used herein refers to increasing in number, as in an increase in the number of T cells. In one embodiment, the T cells that are expanded ex vivo increase in number relative to the number originally present in the culture. In another embodiment, the T cells that are expanded ex vivo increase in number relative to other cell types in the culture. The term "ex vivo," as used herein, refers to cells that have been removed from a living organism, (e.g., a human) and propagated outside the organism (e.g., in a culture dish, test tube, or bioreactor).

The term "expression" as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its promoter.

"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., Sendai viruses, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

"Identity" as used herein refers to the subunit sequence identity between two polymeric molecules particularly between two amino acid molecules, such as, between two polypeptide molecules. When two amino acid sequences have the same residues at the same positions; e.g., if a position in each of two polypeptide molecules is occupied by an arginine, then they are identical at that position. The identity or extent to which two amino acid sequences have the same residues at the same positions in an alignment is often expressed as a percentage. The identity between two amino acid sequences is a direct function of the number of matching or identical positions; e.g., if half (e.g., five positions in a polymer ten amino acids in length) of the positions in two sequences are identical, the two sequences are 50% identical; if 90% of the positions (e.g., 9 of 10), are matched or identical, the two amino acids sequences are 90% identical.

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.

The term "immune response" as used herein is defined as a cellular response to an antigen that occurs when lymphocytes identify antigenic molecules as foreign and induce the formation of antibodies and/or activate lymphocytes to remove the antigen.

The term "immunostimulatory" is used herein to refer to increasing overall immune response.

The term "immunosuppressive" is used herein to refer to reducing overall immune response.

"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.

A "lentivirus" as used herein refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into

the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer in vivo.

The term "limited toxicity" as used herein, refers to the peptides, polynucleotides, cells and/or antibodies of the invention manifesting a lack of substantially negative biological effects, anti-tumor effects, or substantially negative physiological symptoms toward a healthy cell, non-tumor cell, non-diseased cell, non-target cell or population of such cells either in vitro or in vivo.

By the term "modified" as used herein, is meant a changed state or structure of a molecule or cell of the invention. Molecules may be modified in many ways, including chemically, structurally, and functionally. Cells may be modified through the introduction of nucleic acids.

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 and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

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

"Octreotide" is an octapeptide that mimics natural somatostatin. It is a long-acting analog of somatostatin. It is sold under the brand name Sandostatin (Novartis Pharmaceuticals). d-Phe-Cys-Phe-d-Trp-Lys-Thr-Cys-Thr-ol (SEQ ID NO: 40)

"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 term "polynucleotide" as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into the monomeric

"nucleotides." The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum

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number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

The term "self-antigen" as used herein is defined as an antigen that is expressed by a host cell or tissue. Self-antigens may be tumor antigens, but in certain embodiments, are expressed in both normal and tumor cells. A skilled artisan would readily understand that a self-antigen may be overexpressed in a cell.

By the term "specifically binds," 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.

By the term "stimulation," is meant a primary response induced by binding of a stimulatory molecule (e.g., a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex. Stimulation can mediate altered expression of certain molecules, such as downregulation of TGF-beta, and/or reorganization of cytoskeletal structures, and the like.

A "stimulatory molecule," as the term is used herein, means a molecule on a T cell that specifically binds with a cognate stimulatory ligand present on an antigen presenting cell.

A "stimulatory ligand," as used herein, means a ligand that when present on an antigen presenting cell (e.g., an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a "stimulatory molecule") on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands are well-known in the art and encompass, inter alia, an MHC Class I molecule loaded

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with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

The term "subject" is intended to include living organisms in which an immune response can be elicited (e.g., mammals). A "subject" or "patient," as used therein, may be a human or non-human mammal. Non-human mammals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and murine mammals. Preferably, the subject is human.

As used herein, a "substantially purified" cell is a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some embodiments, the cells are cultured *in vitro*. In other embodiments, the cells are not cultured *in vitro*.

As used herein, a switchable CAR (sCAR) refers to a CAR comprising a Peptide-Neo-Epitope (PNE) binding domain, a transmembrane domain, and an intracellular domain.

A "target site" or "target sequence" refers to a genomic nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule may specifically bind under conditions sufficient for binding to occur.

As used herein, the term "T cell receptor" or "TCR" refers to a complex of membrane proteins that participate in the activation of T cells in response to the presentation of antigen. The TCR is responsible for recognizing antigens bound to major histocompatibility complex molecules. TCR is composed of a heterodimer of an alpha ( $\alpha$ ) and beta ( $\beta$ ) chain, although in some cells the TCR consists of gamma and delta ( $\gamma/\delta$ ) chains. TCRs may exist in alpha/beta and gamma/delta forms, which are structurally similar but have distinct anatomical locations and functions. Each chain is composed of two extracellular domains, a variable and constant domain. In some embodiments, the TCR may be modified on any cell comprising a TCR, including, for example, a helper T cell, a cytotoxic T cell, a memory T cell, regulatory T cell, natural killer T cell, and gamma delta T cell.

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.

"Transplant" refers to a biocompatible lattice or a donor tissue, organ or cell, to be transplanted. An example of a transplant may include but is not limited to skin cells or tissue, bone marrow, and solid organs such as heart, pancreas, kidney, lung and liver. A transplant can also refer to any material that is to be administered to a host. For example, a transplant can refer to a nucleic acid or a protein.

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.

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.

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, Sendai viral vectors, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

"Xenogeneic" refers to any material derived from an animal of a different species.

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

#### DESCRIPTION

Acute myeloid leukemia (AML) patients relapsing after chemotherapy have a poor overall 5-year survival prognosis. The present invention provides CAR T cells possessing the necessary specificity towards AML cancer cells while avoiding on-target, off-tumor effects. CD13 is upregulated in AML cells in over 80% of AML patients, but is also moderately expressed in a few types of normal tissues including HSCs. TIM-3 is expressed on the surface of LSCs in many types of human acute myeloid leukemia (AML), but not on hematopoietic stem cells (HSCs). Targeting both TIM3 and CD13, as disclosed herein, provides the specificity needed to target the AML tumor while sparing HSCs and the healthy myeloid compartment of the hemopoietic system.

The present invention includes compositions and methods for bispecific and inducible CAR T cells that specifically kill cancer cells (e.g. AML leukemia stem cells (LSCs)) that commonly express both TIM-3 and CD13.

#### Chimeric Antigen Receptor

Certain embodiments of the invention include chimeric antigen receptors (CARs), including bispecific CARs comprising the following components: At least one antigen binding domain, at least one transmembrane domain, and at least one intracellular domain. The CARs may optionally comprise a hinge domain.

#### a) Antigen Binding Domain

In one embodiment, the CAR (or bispecific CAR) of the invention comprises an antigen binding domain that is a variable domain heavy-chain camelid antibody (VHH), also referred to as a nanobody (Nb). In another embodiment, the CAR comprises an antigen binding domain that binds to TIM-3. In another embodiment, the CAR comprises an antigen binding domain that binds to CD13. In another embodiment, the CAR comprises an antigen binding domain that binds to Peptide-Neo-Epitope (PNE).

The choice of antigen binding domain(s) depends upon the type of CAR (TIM-3 specific, bispecific, or switchable)

being generated. The antigen binding domain(s) may also be chosen depending on the type and number of antigens that are present on the surface of a target cell. For example, the antigen binding domain may be chosen to recognize an antigen that acts as a cell surface marker on a target cell associated with a particular disease state.

The antigen binding domain can include any domain that binds to the antigen and may include, but is not limited to, a nanobody, a monoclonal antibody, a polyclonal antibody, 10 a synthetic antibody, a human antibody, a humanized antibody, a non-human antibody, and any fragment thereof. Thus, in one embodiment, the antigen binding domain portion comprises a mammalian antibody or a fragment thereof.

15 In some instances, the antigen binding domain may be derived from the same species in which the CAR will ultimately be used. For example, for use in humans, the antigen binding domain of the CAR may comprise a human antibody as described elsewhere herein, or a fragment thereof.

20 The antigen binding domain may be operably linked to another domain of the CAR, such as the transmembrane domain or the intracellular domain, both described elsewhere herein, for expression in the cell. In one embodiment, 25 a nucleic acid encoding the antigen binding domain is operably linked to a nucleic acid encoding a transmembrane domain and a nucleic acid encoding an intracellular domain.

20 The antigen binding domains described herein can be combined with any of the transmembrane domains described 30 herein, any of the intracellular domains or cytoplasmic domains described herein, or any of the other domains described herein that may be included in the CAR.

In certain embodiments, the antigen binding domain is nanobody Nb157 (VHH157). In certain embodiments, the 35 antigen binding domain comprises the amino acid sequence of SEQ ID NO: 1. In certain embodiments, the antigen binding domain is encoded by the nucleotide sequence of SEQ ID NO: 2. In certain embodiments, the antigen binding domain comprises a CDR1 sequence comprising the amino acid sequence SYSMA (SEQ ID NO: 3). In certain embodiments, the antigen binding domain comprises a CDR2 sequence comprising the amino acid sequence GIYPSDGKTRYADFKVKGR (SEQ ID NO: 4). In certain 40 embodiments, the antigen binding domain comprises a CDR3 sequence comprising the amino acid sequence ARGITGLGP (SEQ ID NO: 5).

In certain embodiments, the antigen binding domain comprises a TIM-3 binding domain. In certain embodiments, the antigen binding domain comprises an anti-TIM-3 antibody. Antibody molecules to TIM-3 include, but are not limited to, those disclosed in U.S. Pat. No. 9,605,070B2, contents of which are incorporated in their entirety herein. In certain embodiments, the antigen binding domain comprises the amino acid sequence of SEQ ID NO: 6. In certain 50 embodiments, the antigen binding domain is encoded by the nucleotide sequence of SEQ ID NO: 7. In certain embodiments, the antigen binding domain comprises a CDR1 sequence comprising the amino acid sequence of SEQ ID NO: 8. In certain embodiments, the antigen binding domain comprises a CDR2 sequence comprising the amino acid sequence of SEQ ID NO: 9. In certain embodiments, the antigen binding domain comprises a CDR3 sequence comprising the amino acid sequence of SEQ ID NO: 10.

In certain embodiments, the antigen binding domain 55 comprises an anti-TIM-3 nanobody or VHH. In certain embodiments, the nanobody comprises an amino acid sequence selected from the group consisting of SEQ ID NO:

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20, 22, 24, 26, 28, 30, and 32. In certain embodiments, the nanobody is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 19, 21, 23, 25, 27, 29, and 31.

In certain embodiments, the antigen binding domain comprises a PNE-binding domain.

In certain embodiments, the antigen binding domain comprises the amino acid sequence of SEQ ID NO: 11. In certain embodiments, the antigen binding domain is encoded by the nucleotide sequence of SEQ ID NO: 12.

Tolerable variations of the antigen binding domain will be known to those of skill in the art, while maintaining specific binding to the antigen. For example, in some embodiments the antigen binding domain comprises an amino acid sequence that has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to any of the amino acid sequences set forth in SEQ ID NOS: 1, 3-6, 8-11, 20, 22, 24, 26, 28, 30, and 32. In some embodiments the antigen binding domain is encoded by a nucleic acid sequence that has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 2, 7, 12, 19, 21, 23, 25, 27, 29, and 31.

Amino acid sequence of Nb 157

(SEQ ID NO: 1)

AAQAAQVQLQESGGGLVQPGGSLSLSCASGTFSSYSMAWVRQAPKG  
PEWVSGIYPSDGKTRYADFVKGRFSISRDNAKNMLYLQMNNLEPEDTAL  
YYCARGITGLGPRGQGTQVTVSSAA

Nucleotide sequence of Nb 157

(SEQ ID NO: 2)

ggggccccagggtcagctgcaggagtctggggaggcttggcagccctg  
gggggtctctgagccttcctgtacgcctctggattcacgttcagtag  
ttactccatggctgggtccggcaggctccaggaaaggaccgaatgg  
gtctcagggatttacccttctgtatggtaagacaaggatgcagactcg  
tgaaggccgattcagcatctccagagacaacgcacaatgttgta  
tctgcaaatgaacaacctggAACCTGAGGACACGCCATATTACTGT  
ggagaggtatcacggattgggaccccccggccaggggaccaggta  
ccgtctctcagccggcc

Amino acid sequence of TIM-3 scFv

(SEQ ID NO: 6)

QVQLQQPGAEVLVKPGASVKMSCKASGYTFTSYNMHWIKQTPQGLEWIG  
DIYPGNQDTSYNQFKKGATLTADKSSSTVYMQLSSLTSEDSAVYYCAR  
VGGAFPMDYWGQGTSVTVSSGGGSGGGSGGGSGGGSDIVLTQSPA  
SLAVSLGQRATISCRASESVEYYGTSLMQWYQQKPGQPPKLLIYAASNV  
ESGVPARFSGSGSGTDFSLNIHPVEEDDIAIYFCQOSRKDPSTFGGT  
LEIK

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Nucleotide sequence of TIM-3 scFv

(SEQ ID NO: 7)

CAGGTGCAACTGCAGCAGCCTGGGCTGAGCTGGTGAAGCCTGGGCCT  
5 CAGTGAAGATGTCCTGCAAGGCTCTGGCTACACATTACAGTTACAA  
TATGCACTGGATAAAGCAGACACCTGGACAGGGCTGGAATGGATTGGA  
10 GATATTATCCAGGAATGGTATACTTCCTACAATCAGAAATTCAAAG  
GCAAGGGCACATTGACTGCAGACAAATCCTCCAGCACAGTCTACATGCA  
GCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGA  
15 GTGGGGGGTGCCTTCCATGGACTACTGGGTCAGGAACCTCAGTCA  
CCGTCTCCTCAGGAGGGGGAGGATCTGGCGCGAGGAAGTGGCGGAGG  
GGGATCAGGGGGAGGCGGATCTGACATTGTGCTCACCCAATCTCCAGCT  
TCTTGCTGTGCTCTAGGGCAGAGGCCACCATCTCTGAGGCCA  
20 GTGAAAGTGTGAATTATGGCACAAGTTAATGCGAGTGGTACCAA  
GAAACCAGGACAGCCACCCAACTCCTCATCTATGCTGCATCCAACGTA  
GAATCTGGGTCCCTGGCAGGTTAGTGGCAGTGGCTGGGACAGACT  
25 TCAGCCTCAACATCCATCCTGTGGAGGAGGATGATATTGCAATATATT  
CTGTCAGCAAAGTAGGAAGGATCCTTCGACGTTGGAGGACCAAG  
CTGGAGATCAAA

TIM-3 CDR1 AA sequence

(SEQ ID NO: 8)

GYTFTSYNMH

TIM-3 CDR2 AA sequence

(SEQ ID NO: 9)

DIYPGNQDTSYNQFKKG

TIM-3 CDR3 AA sequence

(SEQ ID NO: 10)

VGGAFPMDY

PNE scFv AA sequence

(SEQ ID NO: 11)

HAARPAVVTQESALTSSPGETVTLTCRSSTGAVTTSNYASWVQEKP  
LFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCVL  
YSDHWVFGGGTKLTVLGGGGSGGGSGGGSDVQLQESGPGLV  
45 APSQSLSICTVSGFLTDYGVNWVRQSPGKGLEWLGVIWGDGITDYN  
ALKSRLSVTKDNNSKQVPLKMNSLQSGDSARYCVTGLFDYWQGTT  
VSS

PNE scFv nucleotide sequence

(SEQ ID NO: 12)

CATGCCCTAGACCTGATGCCGTCGTGACCCAGGAAAGCGCCCTGACAA  
GCAGCCCTGGCAGACAGTGCACCTGACCTGACAGATCTAGCACAGGCC  
55 CGTGACCAACAGCAACTACGCCAGCTGGGTGCAGGAAAAGCCGACAC

CTGTTCACGGCCTGATGGCGGCACCAACAATAGAGCACCTGGCGTGC  
CCGCCAGATTCAAGCGGCTCTGTGATCGGAGATAAGGCCGCCCTGACCAT  
60 CACTGGCGCCAGACAGAGGACAGGGCATCTACTTTGCGTGTG  
TACAGCGACCACTGGGTGTTGGCGGAGGGACCAAGCTGACAGTGT  
GCGGAGGCGGAGGATCTGGCGGGAGGAAGTGGCGGAGGGGATCAGG  
65 GGGAGGCGGATCTGATGTGCAAGCTGGCCAGGACTGGTG

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GCCCTAGCCAGAGCCTGAGCATCACCTGTACCGTGTCCGGCTTCCTGC
TGACCGACTACGGCGTGAACCTGGGTGCGCCAGTCTCCTGGCAAGGGCT
GGAATGGCTGGGAGTGTATCTGGGGCAGCGGAATCACCGACTACAACCTC
GCCCTGAAGTCCCGGCTGAGCGTGACCAAGGAAACAGCAAGAGGCCAGG
TGTTCCCTGAAGATGAACAGCCTGCAGAGCGGCCAGCGCCGGTACTA
TTGTGTGACCGGCCTGTTGACTACTGGGCCAGGGCACAAACCTGACC
GTGTCTAGC

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b) Transmembrane Domain

With respect to the transmembrane domain, the CAR (or bispecific CAR) is designed to comprise a transmembrane domain that connects the antigen binding domain of the CAR to the intracellular domain. In one embodiment, the transmembrane domain is naturally associated with one or more of the domains in the CAR. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

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 regions of particular use in this invention may be derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, ICOS, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9.

The transmembrane domains described herein be combined with any of the antigen binding domains described herein, any of the intracellular domains or cytoplasmic domains described herein, or any of the other domains described herein that may be included in the CAR.

In some instances, a variety of hinges can be employed as well including but not limited to the Ig (immunoglobulin) hinge, and the CD8 hinge. The transmembrane domain may be combined with any hinge domain and/or may comprise one or more transmembrane domains described herein. In one embodiment, the transmembrane domain comprises a CD28 transmembrane domain. In another embodiment, the transmembrane domain comprises a CD8 transmembrane domain. In another embodiment, the transmembrane domain comprises a CD8 hinge domain and a CD8 transmembrane domain. In certain embodiments, the hinge domain is selected from the group consisting of a CD8 hinge, an IgG3s hinge, and an IgG4m hinge.

In one embodiment, 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 transmembrane domain.

Between the extracellular domain and the transmembrane domain of the CAR, or between the intracellular domain and the transmembrane domain of the CAR, there may be incorporated a spacer domain. As used herein, the term "spacer domain" generally means any oligo- or polypeptide that functions to link the transmembrane domain to, either the extracellular domain or, the cytoplasmic domain in the polypeptide chain. A spacer domain may comprise up to 300

amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids.

c) Intracellular Domain

The intracellular domain or otherwise the cytoplasmic domain of the CAR (or bispecific CAR) is responsible for activation of the cell in which the CAR is expressed. Examples of an intracellular domain for use in the invention include, but are not limited to, the cytoplasmic portion of a surface receptor, co-stimulatory molecule, and any molecule that acts in concert to initiate signal transduction in the T cell, as well as any derivative or variant of these elements and any synthetic sequence that has the same functional capability.

The intracellular domain of the chimeric membrane protein is responsible for activation of at least one of effector functions of the T cell. While usually the entire intracellular domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The intracellular domain includes any truncated portion of the intracellular domain sufficient to transduce the effector function signal.

In one embodiment, the intracellular domain of the CAR includes any portion of one or more co-stimulatory molecules, such as at least one signaling domain from CD3, CD8, CD27, CD28, ICOS, 4-1BB, PD-1, any derivative or variant thereof, any synthetic sequence thereof that has the same functional capability, and any combination thereof.

Examples of the intracellular domain include a fragment or domain from one or more molecules or receptors including, but are not limited to, TCR, CD3 zeta, CD3 gamma, CD3 delta, CD3 epsilon, CD86, common FcR gamma, FcR beta (Fc Epsilon Rib), CD79a, CD79b, Fcgamma RIIa, DAP10, DAP 12, T cell receptor (TCR), CD8, CD27, CD28, 4-1BB (CD137), OX9, OX40, CD30, CD40, PD-1, ICOS, a KIR family protein, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHT), SLAMF7, NKp80 (KLRF1), CD127, CD 160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD1 Id, ITGAE, CD 103, ITGAL, CD 11a, LFA-1, ITGAM, CD lib, ITGAX, CD 11c, ITGB1, CD29, ITGB2, CD 18, LFA-1, ITGB7, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD 96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD 162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, Toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, other co-stimulatory molecules described herein, any derivative, variant, or fragment thereof, any synthetic sequence of a co-stimulatory molecule that has the same functional capability, and any combination thereof.

The intracellular domains described herein can be combined with any of the antigen binding domains described herein, any of the transmembrane domains described herein, or any of the other domains described herein that may be included in the CAR.

In certain embodiments, the CAR comprises a TIM-3 scFv, an IgG4 hinge region, a CD8 transmembrane domain, a 4-1BB intracellular domain and CD3zeta intracellular domain. In certain embodiments, the CAR comprises the

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amino acid set forth in SEQ ID NO: 13. In certain embodiments, the CAR is encoded by the nucleotide sequence set forth in SEQ ID NO: 14.

Tolerable variations of the CAR sequences will be known to those of skill in the art. For example, in some embodiments the CAR comprises an amino acid sequence that has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the amino acid sequence set forth in SEQ ID NOs: 13. In some embodiments the CAR is encoded by a nucleic acid sequence that has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 33 or 35.

## TIM-3 CAR amino acid sequence

(SEQ ID NO: 13)

MALPV TALLPL ALLLHAARPGSAAQAAQVQLQQPGAE LVKPGASVKMS  
 CKASGYTFTSYNMHWIKQTPGQGLEWIGDIYPGNGDTSYNQFKKGATL  
 TADKSSSTVYMQLSSLTSEDAVYYCARVGGAFPM DYWGQGTSVTVSSG  
 GGGSGGGGGGGGGGGGGGGDIVLTQSPASLA VSLGQRATISCRASESVE  
 YYGTSLMQWYQQKPGQPPKLLIYAASNVESGVPARFSGSGSGTDFSLNI  
 HPVEE DDIAYFCQQRKDPSTFGGGT KLEIKHMGQAGQSGESKYGP  
 PPCPAS YIWAPLAGTCGVLLL S LVI TLYCKRGRKKLLYIFKQPFMRPVQ  
 TTQEEDGCSCRFP EEEEGGCELRVKF SRSDA PAPYKQGQNQLYNELNLG  
 RREEYDVLDKRGRDP EMGGKPRRKNPQEGLYNELQKD KMAEAYSEIGM  
 KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

## TIM-3 CAR nucleotide sequence

(SEQ ID NO: 14)

ATGGCCTTACCA GTGACCGCCTTGCT CCTGCCGCTGGCCTTGCTGCTCC  
 ACGCCGCCAGGCCGGATCCGCCGCCAGGCCGCCAGGTGCAACTGCA  
 GCAGCCTGGGCTGAGCTGGTAAGGCCTGGGCTCAGTGAAGATGTCC  
 TGCAAGGCTTCTGGCTACACATTACAGTTACAATATGCACTGGATAA  
 AGCAGACACCTGGACAGGGCTGGAATGGATTGGAGATTTATCCAGG  
 AAATGGT GATACTT CCTACAATCAGAAATTCAAAGGCAAGGCCACATTG  
 ACTGCAGACAAATCCTCCAGCACAGTCTACATGCAGCTCAGCAGCCTGA  
 CATCTGAGGACTCTGGGTCTATTACTGTGCAAGAGTGGGGGTGCCTT  
 TCCTATGGACTACTGGGTCAAGGAACCTCAGTCACCGTCTCCTCAGGA  
 GCGGGAGGATCTGGCGCGGGAGGAAGTGGCGGAGGGGATCAGGGGGAG  
 GCGGATCTGACATTGTGCTACCCAATCTCAGCTCTTGGCTGTGCT  
 TCTAGGGCAGAGAGCCACCATCTCCTGCAGAGCCAGTGAAGATGTGAA  
 TATTATGGCACAAGTTAATGCAGTGGTACCAACAGAAACCAGGACAGC  
 CACCCAAACTCCTCATCTATGCTGCATCCAACGTAGAATCTGGGTCCC  
 TGCCAGGTTAGTGGCAGTGGGTCTGGGACAGACTTCAGCCTAACATC

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CATCCTGTGGAGGAGGATGATATTGCAATATATTCTGT CAGCAAAGTA  
 GGAAGGATCCTCGACGTT CGGTGGAGGCACCAAGCTGGAGATCAAACAA  
 5 TATGGGCCAGGCCGGCAGTCGGAGAGAGCAAGTACGGCCCTCCCTGC  
 CCCCCCTGCCCCCTGCTAGCTACATCTGGCGCCCTTGGCGGGACTTGTG  
 GGGTCTCTCTCTGTCACTGGTTATCACCTTACTGCCAAACGGGGCAG  
 10 AAAGAAACTCCTGTATATCAAACAAACCATTTAGAGACCAGTACAA  
 ACTACTCAAGAGGAAGATGGCTGTAGCTGCCATTCCAGAAGAAGAAG  
 AAGGAGGATGTGAAC TGA GAGTGAAGTCAGCAGGAGCGCAGACGCC  
 15 CGCGTACAAGCAGGGCAGAACAGCTCTATAACGAGCTCAATCTAGGA  
 CGAAGAGAGGAGTACGATGTTTGGACAAGAGACGTGGCGGGGACCC  
 AGATGGGGGAAAGCCAGAAGGAAGAACCTCAGGAAGGCCTGTACAA  
 20 TGAACTCGAGAAAGATAAGATGGCGGAGGCTACAGTGA GATTGGATG  
 AAAGGCAGCGCCGGAGGGCAAGGGCACGATGGCTTTACCAAGGGTC  
 TCAGTACAGCCACCAAGGACACCTACGACGCCCTCACATGCAGGCC  
 25 GCCCCCTCGCTAA

Included in the invention are isolated polypeptides comprising CARs, isolated nucleic acids comprising CARs, vectors comprising nucleic acids comprising CARs, and modified cells (e.g. T cells) comprising CARs, nucleic acids encoding CARs, or vectors comprising CARs.

## Bispecific CARs

Certain aspects of the invention provide bispecific CARs. A bispecific CAR comprises two different binding specificities and thus binds to two different antigens. In certain embodiments, the bispecific CAR comprises a first antigen binding domain that binds to a first antigen and a second antigen binding domain that binds to a second antigen.

In one aspect, the invention provides a bispecific CAR comprising a first antigen binding domain capable of binding CD13, and a second antigen binding domain capable of binding TIM-3. In certain embodiments, the bispecific CAR comprises a first antigen binding domain capable of binding CD13, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain.

In certain embodiments, the first and/or second antigen binding domain of the bispecific CAR is selected from the group consisting of an antibody, a nanobody, a Fab, an scFv, 50 or any fragment thereof. The antigen binding domains of the bispecific CAR can be combined with any of the transmembrane domains described herein, any of the intracellular domains described herein, any of the hinge domains described herein, or any of the other domains described 55 herein that may be included in the bi-specific CAR.

In certain embodiments, the bispecific CAR comprises a first antigen binding domain comprising a nanobody and a second antigen binding domain comprising an scFv. In certain embodiments, the first antigen binding domain comprising a nanobody is capable of specifically binding CD13 and/or the second antigen binding domain comprising an scFv is capable of specifically binding TIM-3. In certain embodiments, the bi-specific CAR comprises a first antigen binding domain comprising a nanobody capable of binding 60 CD13, a CD3 zeta intracellular domain, a linker, a second antigen binding domain capable of binding TIM-3, a CD28 transmembrane domain, and a 4-1BB intracellular domain.

In certain embodiments, the nanobody capable of binding CD13 is Nb157 and may comprise SEQ ID NO: 1. In certain embodiments, the bi-specific CAR comprises the amino acid sequence set forth in SEQ ID NO: 15, and may be encoded by the nucleotide sequence set forth in SEQ ID NO: 16.

In certain embodiments, the bispecific CAR comprises a first antigen binding domain capable of binding CD13 and a second antigen binding domain capable of binding TIM-3. In certain embodiments, the first antigen binding domain comprises a first nanobody and the second antigen binding domain comprises a second nanobody. In certain embodiments, the second antigen binding domain comprises an anti-TIM-3 nanobody (VHH). In certain embodiments, the CAR comprises an anti-TIM-3 nanobody comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20, 22, 24, 26, 28, 30, and 32. In certain embodiments, the CAR comprises an anti-TIM-3 nanobody encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 19, 21, 23, 25, 27, 29, and 31. In certain embodiments, the bispecific CAR is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs: 33-39.

In certain embodiments, the bispecific CAR may comprise a first nucleic acid sequence encoding a first CAR and a second nucleic acid sequence encoding a second CAR, wherein the first nucleic acid sequence and the second nucleic acid sequence are separated by a linker. Any linker known to one of ordinary skill in the art may be used. In certain embodiments, the linker comprises a cleavage site and/or a self-cleaving peptide. In certain embodiments, the linker is a 2A peptide. In certain embodiments, the 2A peptide is selected from the group consisting of porcine teschovirus-1 2A (P2A), Thoseasigna virus 2A (T2A), equine rhinitis A virus 2A (E2A), and foot-and-mouth disease virus 2A (F2A).

The bispecific CAR, while exhibiting a dual-specificity, may also exhibit split-signaling properties. For example, the a CAR-T cell comprising a TIM-3-specific CAR operably linked to 4-1BBz and a CD13-specific CAR operably linked to CD3z will signal through both 4-1BB and CD3z and synergize to kill target cells when the bispecific CARs bind to the two respective antigens on the cancer cells. In addition, when TIM-3-specific CAR linked to CD3z and a CD13-specific CAR linked to 41BB or CD23/4-1BB domain, they can also synergize to kill the target cells when the target cells express both of the respective cell surface antigens.

In certain embodiments, the bispecific CAR comprises a bispecific antibody. In such embodiments, the bispecific antibody comprises an antigen binding domain comprising a first and a second single chain variable fragment (scFv) molecules. The first and a second scFv are capable of binding two different antigens.

Also provided in the invention is a bispecific CAR comprising an inducible (switchable) element. For example, the bispecific CAR may comprise a switchable CAR (sCAR). In certain embodiments, the sCAR refers to a CAR comprising a Peptide-Neo-Epitope (PNE) binding domain, and optionally a transmembrane domain, and/or an intracellular domain. The switchable CAR can be used in conjunction with a molecule comprising a nanobody fused to a PNE molecule (e.g. a switchable CAR system). When the nanobody-PNE molecule comes into contact with the sCAR, the "switch" is turned on and the CAR T cell is activated.

The nanobody can be fused to the C-terminal region of the PNE or the N-terminal region of the PNE. In certain embodiments, nanobody comprises Nb157 (VHH157) and is fused to the C-terminal region of PNE. In certain embodiments, nanobody is Nb157 and is fused to the N-terminal region of PNE.

In certain embodiments, the inducible bispecific CAR comprises a first antigen binding domain capable of binding Peptide-Neo-Epitope (PNE), a first transmembrane domain, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain. In certain embodiments, the first and/or second transmembrane domain comprises CD28. In certain embodiments, the first intracellular domain comprises CD3 zeta. In certain embodiments, the second intracellular domain comprises 4-1BB. In certain embodiments, the inducible bispecific CAR comprises the amino acid sequence set forth in SEQ ID NO: 17, which may be encoded by the nucleotide sequence set forth in SEQ ID NO: 18.

Included in the invention are isolated polypeptides comprising CARs, isolated nucleic acids comprising CARs, vectors comprising nucleic acids comprising CARs, and modified cells (e.g. T cells) comprising CARs, nucleic acids encoding CARs, or vectors comprising CARs.

Tolerable variations of the CAR sequences will be known to those of skill in the art. For example, in some embodiments the CAR comprises an amino acid sequence that has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to any of the amino acid sequences set forth in SEQ ID NOs: 15 or 17. In some embodiments the CAR is encoded by a nucleic acid sequence that has at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to the nucleic acid sequence set forth in SEQ ID NO: 16 or 18.

## TIM-3&amp;CD13 bispecific CAR amino acid sequence

(SEQ ID NO: 15)

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MALPVALLPLALLLHAARPGSAAQAAQVQLQESGGGLVQPAGGSLSLSCTAGS
FTFSSYSMAWRQAPGKGPEWVSGIYPSDGKTRYADFVKGRFSISRDNAKNMLY
LQMNNLEPEDTALYYCARGITGGLPRGGQTQVTVSSAATSGOTVSSSESKYGPP
CPPCPYIWAFLAGTCGVLLLSLVITLYCRVKFSSRASADAPAYKQGQNOLYNELNLG
RREYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKMAEAYSSEIGMKGER
RRGKGHDGLYQGLSTATKDTYDALHMQLPPLREGSGATNFSSLKQAGDVEEN
PGPPRMALPVALLPLALLLHAARPGSAAQAAQVQLQQPGGAELVKPGASVKMS
CKASGYTFTSYNNMHWIKQTPGQGLEWIGDIYPCNGDTSYQKPKGKATLTADKS
SSTVYMQLSSLTSEDASAVYYCARVGGAFPMWDWGQGTSTVSSGGGGGGGGGS
GGGGGGGGSDIVLTQSPASLAWSLQGRATISCRASESVEYYGTSLMQWYQQKP
GQPPKLLIYAASNVESGVPARFSGSGSGTDFSLNIHPVEEDDIAIFCQOSRKDPST

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FGGGTKEIKHMGKHLCPSPLFPGPSKPFWLVVVGVLACYSLLVTVAIFIIFWV  
 RSKRSRLLHSYDMNMTPRRPGBTTRHYQPYAPPRDFAAYRSKRGRKKLLYIFKQ  
 PFMRPVQTTQEEDGCSRFPEEEAGGCEL

TIM-3&amp;CD13 bispecific CAR nucleotide sequence

(SEQ ID NO: 16)

ATGGCCTTACCACTGACCGCCTTGCTCTGCCGCTGGCCTTGCTGCTCCACGC  
 CGCCAGGCCGGATCCGCGGCCAGCGGCCAGGTGCACTGCAGCTGCAAGGAGTCT  
 GGGGCTGGGCTCTGGTCACTGGCTGGGGCTCTGAGGCTCTCTGTACAGGCC  
 TGGATTACACGTTCACTGAGTACTCATGGCTTGGGCCAGGCTCCAGGGA  
 AGGGACCCGAATGGGTCTCAGGGATTACCCCTCTGATGGTAAGACAAGGTA  
 TGCAGACTTCGTGAAGGCCGATTCACTGCAGACAGACAACGCCAAGAAAT  
 ATGTTGTATCTCAAATGAACACCTGGAACCTGAGGGACAGGCCCTATAATT  
 ACTGTGGAGAGGGTATCACGGATTGGGACCCGGGGCAGGGGACCCAGGT  
 CACCGTCTCTCAGCGGCCACTAGTGGCAGACCGTCTAGGGAGTCTA  
 AGTACGGCCCTCCCTGCCCTCCCTGCCATACATCTGGCGCCCTGGCCGG  
 ACTTGTGGGCTCTTCTCTGTCACTGGTTATCACCTTTACTGAGAGTGA  
 GTTCAGCAGGAGCGAGACGCCCGTACAAGCAGGGCAGAACAGC  
 TATAACGAGCTAACTAGGAGAAGAGGGATCAGTGTGACGATTTGGACAGA  
 GACGTGGCCGGGACCTGAGATGGGGGAAAGCCGAGAAGGAAGAACCTC  
 AGGAAGGCTGTACAATGCAACTGCAAGAAAGATAAGATGGCGAGGCTACA  
 GTGAGATTGGGATGAAAGGCGAGCGCCAGGGCAAGGGGACAGATGGCC  
 TTTTACCAAGGGTCTCAGTACAGGCCAACAGACACTACGACGCCCTCACAT  
 GCAGGCCCTGCCCTCTGCCCTGAGGGAAAGCGAGCTACTAACCTCAGCTG  
 CTGAAGCAGGCTGGAGACGTGGAGGAGAACCTGGACCTCTAGGATGGCT  
 TACCACTGACCCCTTCTGCTCTGCCCTGGCTTGCTGCTCCACGCCGCCAG  
 CGGGGATCCGGCCCAAGGCCAGGTGCAACTGCAGCAGCTGGGCTG  
 AGCTGGTGAAGCTGGGGCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTA  
 CAACATTACCAACTTAAATATGCACTGGATAAGCAGACACCTGGACAGGGC  
 CTGGAATGGGATGGAGATATTATCCAGGAAATGGTGAATCTCTACAAATCA  
 GAATTCAAAGGCCAACGGCACATTGACTGCAGAACATCTCCAGCACAGTC  
 TACATGCAGCTCAGCAGCTGACATCTGAGGACTCTGCGGCTTACTGTGC  
 AAAGAGTGGGGGGTGCCTTCTCTATGGACTCTGGGTCAAGGAACCTCAGTC  
 ACCGGTCTCTCAGGAGGGGGAGATCTGGGGCAGGGAGTGGGGAGGG  
 GGATCAGGGGGGGGGGGATCTGACATCTGCTCACCCAACTCCAGCTTCTT  
 GGCTGTGCTCTAGGGCAGAGGCCACCATCTCTGAGGCCAGTGAAGT  
 GTTGAATATTATGGCACAAGTTAATGCACTGGTACCAACAGAAACCAGGAC  
 AGCCACCCAAACTCCATCTATGCTGATCCAACCTGAGAATCTGGGGTCCCT  
 GCCAGGTTAGGGCAGTGGGTCTGGGACAGACTTCAGCCTCAACATCCATCC  
 TGTTGAGGAGGATGATATTGCAATATATTCTGTCAGCAAAGTAGGAAGGAT  
 CCTCGACGTTGGGGAGGAGGACCAAGCTGGAGATCAAACATATGGGGAAAC  
 ACCTTGTCCAAGTCCCTATTCCCGACCTCTAAGGCCCTTTGGGTCTGG  
 TGGTGGTTGGTGGAGTCTGGGTTGCTATAGCTGCTAGTAACAGTGGCCTT  
 ATTATTTCTGGGGTGGAGTAAGAGGAGCAGGCTCTGCACTGACTACACA  
 TGAACATGACTCCCAGCCGCCCCGGGACCCAGCAAGCATTACAGCCCTA  
 TGCCCAACCCAGGACTTCGCAAGCTTACGCTCCAAACGGGGCAGAAAGAAA  
 CTCTGTATATTCAAACAAACATTATGAGACCAAGTACAACACTACTCAAGA  
 GGAGAGTGGCTAGCTGCCATTCCAGAAGAAGAAGGAGGATGTGA  
 ACTGTAA

TIM-3&amp;PNE inducible bispecific CAR amino acid sequence (FIGS. 8A-8B)

(SEQ ID NO: 17)

MALPVTLALLPLALLLHAARPGSAAQAAQVQLQQPGAEELVKPGASVKMSCKAS  
 GYFTSYNMHWIKQTPQGQLEWIID1YPGNQDTSYNQKFKGKATLTADKSSSTV  
 YMQLSSLTSEDAVYYCARVGGAFPMWDYWGQGTSVTSSGGGGSGGGGSGGG  
 GSGGGGSDIVLQSPASLAVSLGQRATISCRASESVEYYGTSLMOWYQQPKGPQPP  
 KLLIYAASNVESGVPARFSGSGSGTDFSLNIHPVEEDDIAIYFCQSQSRKDPTFQGG  
 TKEIKHMGKHLCPSPLFPGPSKPFWLVVVGVLACYSLLVTVAIFIIFWVRSKR  
 SPLLHSYDMNMTPRRPGBTTRHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMR  
 PVQTTQEEDGCCRFPEEEAGGCELEGGSGATNFSLLKQAGDVNEENPGPPRMALP  
 VTALLPLALLLHAARPDAVVTQESALTSSPGETVLTCTRSSTGAVTTSNYASWV  
 QEKPDHFLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTTITGAQTEDEAIYFCVLW  
 YSDHWVFGGTKLTVLGGGGGGGGGGGSDVQLQESCPGLVAPSQ  
 SLSITCTVSGFLLTDYGVNWRQSPGKGLEWLGIWGDGITDYNALSRSRSLSTVK  
 DNSKSQVFLKMNSLQSGDSARYCVTGLFDYWQGTTLTVSSESKEYGPPCP  
 YIWAPLAGTGVLLSLVITLYCRVFKPSRSADAPAYKQGQNQLYNELNLGRREE  
 DVLDKNGRRDRPEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKGERRRG  
 KGHGDGLYQGLSTATKDTYDALHMQALPPR

TIM-3&amp;PNE inducible bispecific CAR nucleotide sequence (FIGS. 8A-8B)

(SEQ ID NO: 18)

ATGGCCTTACCACTGACCGCCTTGCTCTGCCGCTGGCCTTGCTGCTCCACGC  
 CGCCAGGCCGGATCCGCGGCCAGCGGCCAGGTGCACTGCAGCAGCT  
 GGGGCTGAGCTGGTGAAGCCTGGGGCTCACTGAGATGTCCTGCAAGGCTT  
 CTGGCTACACATTACCAACTATGCACTGGATAAAAGCAGACACCTGG  
 ACAGGGCTGGAAATGGGAGATATTATCAGGAAATGGTGAATCTTCC  
 TACAATCAGAAATTCAAAGGCCACATTGACTGCAGACAACTCTCA  
 GCACAGTCTACATGCAGCTCAGCAGCTGACATCTGAGGACTCTGGGGCTAT  
 TACTGTGCAAGAGTGGGGGTTGCTTCTATGGACTACTGGGGTCAAGGAA  
 CCTCAGTCACCGCTCCTCAGGAGGGGAGGACTCTGGGGGGAGGAGTGG  
 CGGAGGGGGATCAGGGGAGGGGAGTCTGACATTGTGCTCACCCAACTCTCA

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GCTTCTTGGCTGTCTCTAGGGCAGAGACGCCACCATCTCTGCAGAGCCAG  
 TGAAAGTGTGAATATTATGGACAAGTTAATGCACTGGTACCAACAGAAA  
 CCAGGACAGGCCACCAAACCTCTCATCTATGTCATCCAACGTAGAATCTG  
 GGGTCCCTGCCAGGTTAGTGGCAGTGGGCTGGGACAGACTCAGCCTCAA  
 CATCCATCCTGTGGAGGAGATGATATTGCAATATTCTGTCAAGCAAAGTA  
 GGAAGGATCCTCGACGTCGGAGGACCAAAGCTGGAGATCAAACATAT  
 GGGGAAACACCTTTGTCAGTCCCCTATTCCCGACCTCTAACGCCCTTT  
 GGGTGTGGTGGTGGAGTCTGGCTTGCTATAGCTGTAGTAACA  
 GTGGCTTATATTCTGTAGGAGTAAGAGGAGCAGCTCTGACAG  
 TGACTACATGAACATGACTCCCGCCCGGCCACCCGCAAGCATTAC  
 CAGCCCTATGCCACCGCAGCTCGCACCTATCGCTCAAAGGGCA  
 GAAAAGAAACTCTGTATATTCAGAACACATTAGAGACAGTACAAC  
 TAATCAAGAGGAAGATGGCTTAGCTGGCAGTTCAGAAGAGAGAAGGA  
 GGATGTGAACCTCGAGGGAGCAGGGCTACTAACTTCAGCTGTGAAGC  
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 CGCTGTCAGCCAGGAAGCGCCCTGACAACAGCAGCCCTGGCGAGCAGTGAC  
 CCTGACCTGCAAGATCTAGCACAGCGCGTGAACCACAGCAACTACGCCAGC  
 TGGGTGAGGAAAGCCGACACCTGTTACCGGCTGATCGCGGACCCA  
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 GACAGTGCTGGGGAGGGCGATCTGATGTGCACTGTCAGGAATCTGGCCAGGA  
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 GCTGAGGACTACGGCGTGAAGCTGGTGCAGCTCCTGGCAAGGGCTG  
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 TGAAGTCCCGCTGAGCGTACCAAGGACAACAGCAAGAGGCCAGGTGTTCT  
 GAAGATGAACACGCTGAGCGGACAGGCCCGGTACTATTGTGTGACC  
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 GCTCTATAACAGCTCAATCTAGGACAGAAGAGGAGTACGATGTTTGAC  
 AAGAGACGTGGGGACCTGAGATGGGGAAAGCCGAGAGGAAC  
 CCTCAGGAAGGGCTGTACAATGAACATGCAAGAAAGATAAGTGGCGAGGCT  
 ACAGTGAGATTGGATGAAAGCGAGGCCGGAGGGCAAGGGCACGATG  
 GCCTTACACGAGCTCTAGTACAGCACCAAGGACACCTACGACGCCCTCA  
 CATGCAGGCCCTGCCCTCGCTAA

B1CAR2 VHH12

(SEQ ID NO: 33)

GTGACAGAGTGGTTACATGAACTGGATCTAACAGCGGTAAAGATCTTG  
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 CATGAGTGATAACACTCGGCCAACCTACTCTGACAACGATCGGAGGACCG  
 AAGGAGCTAACCGCTTTTGACAACATGGGGATCATGTAACTCGCCTG  
 TCGTTGGGAACCGGAGCTGAAATGAAGGCACTAACAAACGACGGAGCTGACACC  
 ACAGATGCGCTGAGCAATGGCAACACGTTGCGCAAACATTAAACTGGCGAAC  
 TACTTACTCTACCTCCCGGACAACATTAAAGACTGGATGGAGGGAGATAA  
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 CCGTAGAGAAAAGTCAAAGGATCTTCTGGAGATCCTTTTCTGGCTAAC  
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 GGCACCTCGATTAGTTCTGCTTGGAGTAGCTGCTTGTAGTTGGGG  
 GAGGGGGTTATGGCATGGAGTTCCCCACACTGAGTAGGGTGAGACTGAAG  
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 AGCGCCCAGACCACTACCCGGCTCTACCATCGATCTCAGCCCTTGAGTC  
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 TTGCACTTATAATGGTTACAAATAAGCAATAGCATCACAATT  
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 TCCCTTCGCCACGTTGCCGCTTCCGCTCAAGCTTAATGGGG  
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(SEQ ID NO: 34)

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(SEQ ID NO: 35)

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(SEQ ID NO: 37)

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(SEQ ID NO: 38)

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(SEQ ID NO: 39)

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ATCAGTTGG

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#### Antibodies and Nanobodies

In certain aspects, the invention provides anti-TIM3 antibodies and nanobodies. In certain embodiments, the antibody or nanobody comprises an amino acid sequence at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any of the amino acid sequences set forth in SEQ ID NO: 20, 22, 24, 26, 28, 30, and 32. In certain embodiments, the antibody or nanobody is encoded by a nucleotide sequence at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to any of the nucleotide sequences set forth in SEQ ID NO: 19, 21, 23, 25, 27, 29, and 31.

#### Modified Immune Cells

The present invention provides modified immune cells or precursors thereof (e.g., T cells) comprising chimeric antigen receptors (CARs) or bispecific CARs capable of binding TIM-3. Also provided are modified immune cells or precursors thereof (e.g., T cells) comprising bispecific CARs capable of binding TIM-3 and CD13. The invention includes modified immune cells or precursors thereof comprising any of the CARs or bispecific CARs disclosed herein. The invention also includes modified immune cells or precursors thereof comprising any of the nucleic acids disclosed herein or any of the vectors disclosed herein.

In certain embodiments, the modified cell is a modified immune cell. In certain embodiments, the modified cell is a modified T cell. In certain embodiments, the modified cell is an autologous cell. In certain embodiments, the modified cell is an autologous cell obtained from a human subject.

#### Methods of Treatment

The modified cells (e.g., CAR T cells) described herein, may be included in a composition for immunotherapy. The composition may include a pharmaceutical composition and further include a pharmaceutically acceptable carrier. A therapeutically effective amount of the pharmaceutical composition comprising the modified T cells may be administered.

In one aspect, the invention includes a method for adoptive cell transfer therapy comprising administering to a subject in need thereof a modified T cell of the present

invention. In another aspect, the invention includes a method of treating a disease or condition in a subject comprising administering to a subject in need thereof a population of modified T cells. In certain embodiments, the disease to be treated is cancer. In certain embodiments, the cancer is acute myeloid leukemia (AML).

Methods for administration of immune cells for adoptive cell therapy are known in the art and may be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; U.S. Pat. No. 4,690,915 to Rosenberg; Rosenberg (2011) *Nat Rev Clin Oncol.* 8 (10): 577-85. See, e.g., Themeli et al. (2013) *Nat Biotechnol.* 31 (10): 928-933; Tsukahara et al. (2013) *Biochem Biophys Res Commun* 438 (1): 84-9; Davila et al. (2013) *PLOS ONE* 8 (4): e61338. In some embodiments, the cell therapy, e.g., adoptive T cell therapy is carried out by autologous transfer, in which the cells are isolated and/or otherwise prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some aspects, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells, following isolation and processing are administered to the same subject.

In some embodiments, the cell therapy, e.g., adoptive T cell therapy, is carried out by allogeneic transfer, in which the cells are isolated and/or otherwise prepared from a subject other than a subject who is to receive or who ultimately receives the cell therapy, e.g., a first subject. In such embodiments, the cells then are administered to a different subject, e.g., a second subject, of the same species. In some embodiments, the first and second subjects are genetically identical. In some embodiments, the first and second subjects are genetically similar. In some embodiments, the second subject expresses the same HLA class or supertype as the first subject.

In some embodiments, the subject has been treated with a therapeutic agent targeting the disease or condition, e.g. the tumor, prior to administration of the cells or composition containing the cells. In some aspects, the subject is refractory or non-responsive to the other therapeutic agent. In some embodiments, the subject has persistent or relapsed disease, e.g., following treatment with another therapeutic intervention, including chemotherapy, radiation, and/or

hematopoietic stem cell transplantation (HSCT), e.g., allogenic HSCT. In some embodiments, the administration effectively treats the subject despite the subject having become resistant to another therapy.

In some embodiments, the subject is responsive to the other therapeutic agent, and treatment with the therapeutic agent reduces disease burden. In some aspects, the subject is initially responsive to the therapeutic agent, but exhibits a relapse of the disease or condition over time. In some embodiments, the subject has not relapsed. In some such embodiments, the subject is determined to be at risk for relapse, such as at a high risk of relapse, and thus the cells are administered prophylactically, e.g., to reduce the likelihood of or prevent relapse. In some aspects, the subject has not received prior treatment with another therapeutic agent.

The modified immune cells of the present invention can be administered to an animal, preferably a mammal, even more preferably a human, to treat a cancer (e.g. AML). In addition, the cells of the present invention can be used for the treatment of any condition related to a cancer, especially a cell-mediated immune response against a tumor cell(s), where it is desirable to treat or alleviate the disease. The types of cancers to be treated with the modified cells or pharmaceutical compositions of the invention include, acute myeloid leukemia, chronic myeloid leukemia, pancreatic neuroendocrine tumor (PNETs), gastrointestinal NETs, and lung and prostate cancer NETs, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Other exemplary cancers include but are not limited breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer, thyroid cancer, and the like. The cancers may be non-solid tumors (such as hematological tumors) or solid tumors. Adult tumors/cancers and pediatric tumors/cancers are also included. In one embodiment, the cancer is a solid tumor or a hematological tumor. In one embodiment, the cancer is a carcinoma. In one embodiment, the cancer is a sarcoma. In one embodiment, the cancer is a leukemia. In one embodiment the cancer is a solid tumor. In one embodiment, the cancer is ovarian cancer. In one embodiment, the cancer is endometrial cancer. In one embodiment, the cancer is AML.

Solid tumors are abnormal masses of tissue that usually do not contain cysts or liquid areas. Solid tumors can be benign or malignant. Different types of solid tumors are named for the type of cells that form them (such as sarcomas, carcinomas, and lymphomas). Examples of solid tumors, such as sarcomas and carcinomas, include fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteosarcoma, and other sarcomas, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, lymphoid malignancy, pancreatic cancer, breast cancer, lung cancers, ovarian cancer, prostate cancer, hepatocellular carcinoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, medullary thyroid carcinoma, papillary thyroid carcinoma, pheochromocytoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, Wilms' tumor, cervical cancer, testicular tumor, seminoma, bladder carcinoma, melanoma, and CNS tumors (such as a glioma (such as brainstem glioma and mixed gliomas), glioblastoma (also known as glioblastoma multiforme) astrocytoma, CNS lymphoma, germinoma, medulloblastoma, Schwannoma cran-

iopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendrogloma, menangioma, neuroblastoma, retinoblastoma and brain metastases).

Carcinomas that can be amenable to therapy by a method disclosed herein include, but are not limited to, esophageal carcinoma, hepatocellular carcinoma, basal cell carcinoma (a form of skin cancer), squamous cell carcinoma (various tissues), bladder carcinoma, including transitional cell carcinoma (a malignant neoplasm of the bladder), bronchogenic carcinoma, colon carcinoma, colorectal carcinoma, gastric carcinoma, lung carcinoma, including small cell carcinoma and non-small cell carcinoma of the lung, adrenocortical carcinoma, thyroid carcinoma, pancreatic carcinoma, breast carcinoma, ovarian carcinoma, prostate carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, renal cell carcinoma, ductal carcinoma in situ or bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical carcinoma, uterine carcinoma, testicular carcinoma, osteogenic carcinoma, epithelial carcinoma, and nasopharyngeal carcinoma, ovarian cancer, endometrial cancer, uterine sarcoma, cervical carcinoma, breast cancer, lung cancer, prostate cancer, ocular melanoma, and any MISIIR-expressing tumor.

Sarcomas that can be amenable to therapy by a method disclosed herein include, but are not limited to, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, chordoma, osteogenic sarcoma, osteosarcoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's sarcoma, leiomyosarcoma, rhabdomyosarcoma, and other soft tissue sarcomas.

In certain exemplary embodiments, the modified immune cells of the invention are used to treat a myeloma, or a condition related to myeloma. Examples of myeloma or conditions related thereto include, without limitation, light chain myeloma, non-secretory myeloma, monoclonal gammopathy of undetermined significance (MGUS), plasmacytoma (e.g., solitary, multiple solitary, extramedullary plasmacytoma), amyloidosis, and multiple myeloma. In one embodiment, a method of the present disclosure is used to treat multiple myeloma. In one embodiment, a method of the present disclosure is used to treat refractory myeloma. In one embodiment, a method of the present disclosure is used to treat relapsed myeloma.

In certain exemplary embodiments, the modified immune cells of the invention are used to treat a melanoma, or a condition related to melanoma. Examples of melanoma or conditions related thereto include, without limitation, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, amelanotic melanoma, or melanoma of the skin (e.g., cutaneous, eye, vulva, vagina, rectum melanoma). In one embodiment, a method of the present disclosure is used to treat cutaneous melanoma. In one embodiment, a method of the present disclosure is used to treat refractory melanoma. In one embodiment, a method of the present disclosure is used to treat relapsed melanoma.

In yet other exemplary embodiments, the modified immune cells of the invention are used to treat a sarcoma, or a condition related to sarcoma. Examples of sarcoma or conditions related thereto include, without limitation, angiosarcoma, chondrosarcoma, Ewing's sarcoma, fibrosarcoma, gastrointestinal stromal tumor, leiomyosarcoma, liposarcoma, malignant peripheral nerve sheath tumor, osteosarcoma, pleomorphic sarcoma, rhabdomyosarcoma,

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and synovial sarcoma. In one embodiment, a method of the present disclosure is used to treat synovial sarcoma. In one embodiment, a method of the present disclosure is used to treat liposarcoma such as myxoid/round cell liposarcoma, differentiated/dedifferentiated liposarcoma, and pleomorphic liposarcoma. In one embodiment, a method of the present disclosure is used to treat myxoid/round cell liposarcoma. In one embodiment, a method of the present disclosure is used to treat a refractory sarcoma. In one embodiment, a method of the present disclosure is used to treat a relapsed sarcoma.

The cells of the invention to be administered may be autologous, with respect to the subject undergoing therapy.

The administration of the cells of the invention may be carried out in any convenient manner known to those of skill in the art. The cells of the present invention may be administered to a subject by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient transarterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In other instances, the cells of the invention are injected directly into a site of inflammation in the subject, a local disease site in the subject, a lymph node, an organ, a tumor, and the like.

In some embodiments, the cells are administered at a desired dosage, which in some aspects includes a desired dose or number of cells or cell type(s) and/or a desired ratio of cell types. Thus, the dosage of cells in some embodiments is based on a total number of cells (or number per kg body weight) and a desired ratio of the individual populations or sub-types, such as the CD4+ to CD8+ ratio. In some embodiments, the dosage of cells is based on a desired total number (or number per kg of body weight) of cells in the individual populations or of individual cell types. In some embodiments, the dosage is based on a combination of such features, such as a desired number of total cells, desired ratio, and desired total number of cells in the individual populations.

In some embodiments, the populations or sub-types of cells, such as CD8+ and CD4+ T cells, are administered at or within a tolerated difference of a desired dose of total cells, such as a desired dose of T cells. In some aspects, the desired dose is a desired number of cells or a desired number of cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some aspects, the desired dose is at or above a minimum number of cells or minimum number of cells per unit of body weight. In some aspects, among the total cells, administered at the desired dose, the individual populations or sub-types are present at or near a desired output ratio (such as CD4+ to CD8+ ratio), e.g., within a certain tolerated difference or error of such a ratio.

In some embodiments, the cells are administered at or within a tolerated difference of a desired dose of one or more of the individual populations or sub-types of cells, such as a desired dose of CD4+ cells and/or a desired dose of CD8+ cells. In some aspects, the desired dose is a desired number of cells of the sub-type or population, or a desired number of such cells per unit of body weight of the subject to whom the cells are administered, e.g., cells/kg. In some aspects, the desired dose is at or above a minimum number of cells of the population or subtype, or minimum number of cells of the population or sub-type per unit of body weight. Thus, in some embodiments, the dosage is based on a desired fixed dose of total cells and a desired ratio, and/or based on a desired fixed dose of one or more, e.g., each, of the individual sub-types or sub-populations. Thus, in some embodi-

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ments, the dosage is based on a desired fixed or minimum dose of T cells and a desired ratio of CD4+ to CD8+ cells, and/or is based on a desired fixed or minimum dose of CD4+ and/or CD8+ cells.

In certain embodiments, the cells, or individual populations of sub-types of cells, are administered to the subject at a range of about one million to about 100 billion cells, such as, e.g., 1 million to about 50 billion cells (e.g., about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (e.g., about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (e.g., about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges.

In some embodiments, the dose of total cells and/or dose of individual sub-populations of cells is within a range of between at or about  $1 \times 10^5$  cells/kg to about  $1 \times 10^{11}$  cells/kg  $10^4$  and at or about  $10^{11}$  cells/kilograms (kg) body weight, such as between  $10^5$  and  $10^6$  cells/kg body weight, for example, at or about  $1 \times 10^5$  cells/kg,  $1.5 \times 10^5$  cells/kg,  $2 \times 10^5$  cells/kg, or  $1 \times 10^6$  cells/kg body weight. For example, in some embodiments, the cells are administered at, or within a certain range of error of, between at or about  $10^4$  and at or about  $10^9$  T cells/kilograms (kg) body weight, such as between  $10^5$  and  $10^6$  T cells/kg body weight, for example, at or about  $1 \times 10^5$  T cells/kg,  $1.5 \times 10^5$  T cells/kg,  $2 \times 10^5$  T cells/kg, or  $1 \times 10^6$  T cells/kg body weight. In other exemplary embodiments, a suitable dosage range of modified cells for use in a method of the present disclosure includes, without limitation, from about  $1 \times 10^5$  cells/kg to about  $1 \times 10^6$  cells/kg, from about  $1 \times 10^6$  cells/kg to about  $1 \times 10^7$  cells/kg, from about  $1 \times 10^7$  cells/kg about  $1 \times 10^8$  cells/kg, from about  $1 \times 10^8$  cells/kg about  $1 \times 10^9$  cells/kg, from about  $1 \times 10^9$  cells/kg about  $1 \times 10^{10}$  cells/kg, from about  $1 \times 10^{10}$  cells/kg about  $1 \times 10^{11}$  cells/kg. In an exemplary embodiment, a suitable dosage for use in a method of the present disclosure is about  $1 \times 10^8$  cells/kg. In an exemplary embodiment, a suitable dosage for use in a method of the present disclosure is about  $1 \times 10^7$  cells/kg. In other embodiments, a suitable dosage is from about  $1 \times 10^7$  total cells to about  $5 \times 10^7$  total cells. In some embodiments, a suitable dosage is from about  $1 \times 10^8$  total cells to about  $5 \times 10^8$  total cells. In some embodiments, a suitable dosage is from about  $1.4 \times 10^7$  total cells to about  $1.1 \times 10^9$  total cells. In an exemplary embodiment, a suitable dosage for use in a method of the present disclosure is about  $7 \times 10^9$  total cells.

In some embodiments, the cells are administered at or within a certain range of error of between at or about  $10^4$  and at or about  $10^9$  CD4+ and/or CD8+ cells/kilograms (kg) body weight, such as between  $10^5$  and  $10^6$  CD4+ and/or CD8+ cells/kg body weight, for example, at or about  $1 \times 10^5$  CD4+ and/or CD8+ cells/kg,  $1.5 \times 10^5$  CD4+ and/or CD8+ cells/kg,  $2 \times 10^5$  CD4+ and/or CD8+ cells/kg, or  $1 \times 10^6$  CD4+ and/or CD8+ cells/kg body weight. In some embodiments, the cells are administered at or within a certain range of error of, greater than, and/or at least about  $1 \times 10^6$ , about  $2.5 \times 10^6$ , about  $5 \times 10^6$ , about  $7.5 \times 10^6$ , or about  $9 \times 10^6$  CD4+ cells,

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and/or at least about  $1\times 10^6$ , about  $2.5\times 10^6$ , about  $5\times 10^6$ , about  $7.5\times 10^6$ , or about  $9\times 10^6$  CD8<sup>+</sup> cells, and/or at least about  $1\times 10^6$ , about  $2.5\times 10^6$ , about  $5\times 10^6$ , about  $7.5\times 10^6$ , or about  $9\times 10^6$  T cells. In some embodiments, the cells are administered at or within a certain range of error of between about  $10^8$  and  $10^{12}$  or between about  $10^{10}$  and  $10^{11}$  T cells, between about  $10^8$  and  $10^{12}$  or between about  $10^{10}$  and  $10^{11}$  CD4<sup>+</sup> cells, and/or between about  $10^8$  and  $10^{12}$  or between about  $10^{10}$  and  $10^{11}$  CD8<sup>+</sup> cells.

In some embodiments, the cells are administered at or within a tolerated range of a desired output ratio of multiple cell populations or sub-types, such as CD4<sup>+</sup> and CD8<sup>+</sup> cells or sub-types. In some aspects, the desired ratio can be a specific ratio or can be a range of ratios, for example, in some embodiments, the desired ratio (e.g., ratio of CD4<sup>+</sup> to CD8<sup>+</sup> cells) is between at or about 5:1 and at or about 5:1 (or greater than about 1:5 and less than about 5:1), or between at or about 1:3 and at or about 3:1 (or greater than about 1:3 and less than about 3:1), such as between at or about 2:1 and at or about 1:5 (or greater than about 1:5 and less than about 2:1, such as at or about 5:1, 4.5:1, 4:1, 3.5:1, 3:1, 2.5:1, 2:1, 1.9:1, 1.8:1, 1.7:1, 1.6:1, 1.5:1, 1.4:1, 1.3:1, 1.2:1, 1.1:1, 1:1, 1:1.1, 1:1.2, 1:1.3, 1:1.4, 1:1.5, 1:1.6, 1:1.7, 1:1.8, 1:1.9: 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, or 1:5. In some aspects, the tolerated difference is within about 1%, about 2%, about 3%, about 4% about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50% of the desired ratio, including any value in between these ranges.

In some embodiments, a dose of modified cells is administered to a subject in need thereof, in a single dose or multiple doses. In some embodiments, a dose of modified cells is administered in multiple doses, e.g., once a week or every 7 days, once every 2 weeks or every 14 days, once every 3 weeks or every 21 days, once every 4 weeks or every 28 days. In an exemplary embodiment, a single dose of modified cells is administered to a subject in need thereof. In an exemplary embodiment, a single dose of modified cells is administered to a subject in need thereof by rapid intravenous infusion.

For the prevention or treatment of disease, the appropriate dosage may depend on the type of disease to be treated, the type of cells or recombinant receptors, the severity and course of the disease, whether the cells are administered for preventive or therapeutic purposes, previous therapy, the subject's clinical history and response to the cells, and the discretion of the attending physician. The compositions and cells are in some embodiments suitably administered to the subject at one time or over a series of treatments.

In some embodiments, the cells are administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention, such as an antibody or engineered cell or receptor or agent, such as a cytotoxic or therapeutic agent. The cells in some embodiments are co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or more additional therapeutic agents, or vice versa. In some embodiments, the cells are administered prior to the one or more additional therapeutic agents. In some embodiments, the cells are administered after the one or more additional therapeutic agents. In some embodiments, the one or more additional agents include a cytokine, such as IL-2, for

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example, to enhance persistence. In some embodiments, the methods comprise administration of a chemotherapeutic agent.

In certain embodiments, the modified cells of the invention (e.g., a modified cell comprising a CAR) may be administered to a subject in combination with an immune checkpoint antibody (e.g., an anti-PD1, anti-CTLA-4, or anti-PDL1 antibody). For example, the modified cell may be administered in combination with an antibody or antibody fragment targeting, for example, PD-1 (programmed death 1 protein). Examples of anti-PD-1 antibodies include, but are not limited to, pembrolizumab (KEYTRUDA®, formerly lambrolizumab, also known as MK-3475), and nivolumab (BMS-936558, MDX-1106, ONO-4538, OPDIVA®) or an antigen-binding fragment thereof. In certain embodiments, the modified cell may be administered in combination with an anti-PD-L1 antibody or antigen-binding fragment thereof. Examples of anti-PD-L1 antibodies include, but are not limited to, BMS-936559, MPDL3280A (TECENTRIQ®, Atezolizumab), and MEDI4736 (Durvalumab, Imfinzi). In certain embodiments, the modified cell may be administered in combination with an anti-CTLA-4 antibody or antigen-binding fragment thereof. An example of an anti-CTLA-4 antibody includes, but is not limited to, Ipilimumab (trade name Yervoy). Other types of immune checkpoint modulators may also be used including, but not limited to, small molecules, siRNA, miRNA, and CRISPR systems. Immune checkpoint modulators may be administered before, after, or concurrently with the modified cell comprising the CAR. In certain embodiments, combination treatment comprising an immune checkpoint modulator may increase the therapeutic efficacy of a therapy comprising a modified cell of the present invention.

Following administration of the cells, the biological activity of the engineered cell populations in some embodiments is measured, e.g., by any of a number of known methods. Parameters to assess include specific binding of an engineered or natural T cell or other immune cell to antigen, in vivo, e.g., by imaging, or ex vivo, e.g., by ELISA or flow cytometry. In certain embodiments, the ability of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer et al., J. Immunotherapy, 32 (7): 689-702 (2009), and Herman et al. J. Immunological Methods, 285 (1): 25-40 (2004). In certain embodiments, the biological activity of the cells is measured by assaying expression and/or secretion of one or more cytokines, such as CD 107a, IFN $\gamma$ , IL-2, and TNF. In some aspects the biological activity is measured by assessing clinical outcome, such as reduction in tumor burden or load.

In certain embodiments, the subject is provided a secondary treatment. Secondary treatments include but are not limited to chemotherapy, radiation, surgery, and medications.

In some embodiments, the subject can be administered a conditioning therapy prior to CAR T cell therapy. In some embodiments, the conditioning therapy comprises administering an effective amount of cyclophosphamide to the subject. In some embodiments, the conditioning therapy comprises administering an effective amount of fludarabine to the subject. In preferred embodiments, the conditioning therapy comprises administering an effective amount of a combination of cyclophosphamide and fludarabine to the subject. Administration of a conditioning therapy prior to CAR T cell therapy may increase the efficacy of the CAR T cell therapy. Methods of conditioning patients for T cell

therapy are described in U.S. Pat. No. 9,855,298, which is incorporated herein by reference in its entirety.

In some embodiments, a specific dosage regimen of the present disclosure includes a lymphodepletion step prior to the administration of the modified T cells. In an exemplary embodiment, the lymphodepletion step includes administration of cyclophosphamide and/or fludarabine.

In some embodiments, the lymphodepletion step includes administration of cyclophosphamide at a dose of between about 200 mg/m<sup>2</sup>/day and about 2000 mg/m<sup>2</sup>/day (e.g., 200 mg/m<sup>2</sup>/day, 300 mg/m<sup>2</sup>/day, or 500 mg/m<sup>2</sup>/day). In an exemplary embodiment, the dose of cyclophosphamide is about 300 mg/m<sup>2</sup>/day. In some embodiments, the lymphodepletion step includes administration of fludarabine at a dose of between about 20 mg/m<sup>2</sup>/day and about 900 mg/m<sup>2</sup>/day (e.g., 20 mg/m<sup>2</sup>/day, 25 mg/m<sup>2</sup>/day, 30 mg/m<sup>2</sup>/day, or 60 mg/m<sup>2</sup>/day). In an exemplary embodiment, the dose of fludarabine is about 30 mg/m<sup>2</sup>/day.

In some embodiment, the lymphodepletion step includes administration of cyclophosphamide at a dose of between about 200 mg/m<sup>2</sup>/day and about 2000 mg/m<sup>2</sup>/day (e.g., 200 mg/m<sup>2</sup>/day, 300 mg/m<sup>2</sup>/day, or 500 mg/m<sup>2</sup>/day), and fludarabine at a dose of between about 20 mg/m<sup>2</sup>/day and about 900 mg/m<sup>2</sup>/day (e.g., 20 mg/m<sup>2</sup>/day, 25 mg/m<sup>2</sup>/day, 30 mg/m<sup>2</sup>/day, or 60 mg/m<sup>2</sup>/day). In an exemplary embodiment, the lymphodepletion step includes administration of cyclophosphamide at a dose of about 300 mg/m<sup>2</sup>/day, and fludarabine at a dose of about 30 mg/m<sup>2</sup>/day.

In an exemplary embodiment, the dosing of cyclophosphamide is 300 mg/m<sup>2</sup>/day over three days, and the dosing of fludarabine is 30 mg/m<sup>2</sup>/day over three days.

Dosing of lymphodepletion chemotherapy may be scheduled on Days -6 to -4 (with a -1 day window, i.e., dosing on Days -7 to -5) relative to T cell (e.g., CAR-T.) infusion on Day 0.

In an exemplary embodiment, for a subject having cancer, the subject receives lymphodepleting chemotherapy including 300 mg/m<sup>2</sup> of cyclophosphamide by intravenous infusion 3 days prior to administration of the modified T cells. In an exemplary embodiment, for a subject having cancer, the subject receives lymphodepleting chemotherapy including 300 mg/m<sup>2</sup> of cyclophosphamide by intravenous infusion for 3 days prior to administration of the modified T cells.

In an exemplary embodiment, for a subject having cancer, the subject receives lymphodepleting chemotherapy including fludarabine at a dose of between about 20 mg/m<sup>2</sup>/day and about 900 mg/m<sup>2</sup>/day (e.g., 20 mg/m<sup>2</sup>/day, 25 mg/m<sup>2</sup>/day, 30 mg/m<sup>2</sup>/day, or 60 mg/m<sup>2</sup>/day). In an exemplary embodiment, for a subject having cancer, the subject receives lymphodepleting chemotherapy including fludarabine at a dose of 30 mg/m<sup>2</sup> for 3 days.

In an exemplary embodiment, for a subject having cancer, the subject receives lymphodepleting chemotherapy including cyclophosphamide at a dose of between about 200 mg/m<sup>2</sup>/day and about 2000 mg/m<sup>2</sup>/day (e.g., 200 mg/m<sup>2</sup>/day, 300 mg/m<sup>2</sup>/day, or 500 mg/m<sup>2</sup>/day), and fludarabine at a dose of between about 20 mg/m<sup>2</sup>/day and about 900 mg/m<sup>2</sup>/day (e.g., 20 mg/m<sup>2</sup>/day, 25 mg/m<sup>2</sup>/day, 30 mg/m<sup>2</sup>/day, or 60 mg/m<sup>2</sup>/day). In an exemplary embodiment, for a subject having cancer, the subject receives lymphodepleting chemotherapy including cyclophosphamide at a dose of about 300 mg/m<sup>2</sup>/day, and fludarabine at a dose of 30 mg/m<sup>2</sup> for 3 days.

Cells of the invention can be administered in dosages and routes and at times to be determined in appropriate pre-clinical and clinical experimentation and trials. Cell com-

positions may be administered multiple times at dosages within these ranges. Administration of the cells of the invention may be combined with other methods useful to treat the desired disease or condition as determined by those of skill in the art.

It is known in the art that one of the adverse effects following infusion of CAR T cells is the onset of immune activation, known as cytokine release syndrome (CRS). CRS is immune activation resulting in elevated inflammatory cytokines. CRS is a known on-target toxicity, development of which likely correlates with efficacy. Clinical and laboratory measures range from mild CRS (constitutional symptoms and/or grade-2 organ toxicity) to severe CRS (sCRS; grade ≥3 organ toxicity, aggressive clinical intervention, and/or potentially life threatening). Clinical features include: high fever, malaise, fatigue, myalgia, nausea, anorexia, tachycardia/hypotension, capillary leak, cardiac dysfunction, renal impairment, hepatic failure, and disseminated intravascular coagulation. Dramatic elevations of cytokines including interferon-gamma, granulocyte macrophage colony-stimulating factor, IL-10, and IL-6 have been shown following CAR T-cell infusion. One CRS signature is elevation of cytokines including IL-6 (severe elevation), IFN-gamma, TNF-alpha (moderate), and IL-2 (mild). Elevations in clinically available markers of inflammation including ferritin and C-reactive protein (CRP) have also been observed to correlate with the CRS syndrome. The presence of CRS generally correlates with expansion and progressive immune activation of adoptively transferred cells. It has been demonstrated that the degree of CRS severity is dictated by disease burden at the time of infusion as patients with high tumor burden experience a more sCRS.

Accordingly, the invention provides for, following the diagnosis of CRS, appropriate CRS management strategies to mitigate the physiological symptoms of uncontrolled inflammation without dampening the antitumor efficacy of the engineered cells (e.g., CAR T cells). CRS management strategies are known in the art. For example, systemic corticosteroids may be administered to rapidly reverse symptoms of sCRS (e.g., grade 3 CRS) without compromising initial antitumor response.

In some embodiments, an anti-IL-6R antibody may be administered. An example of an anti-IL-6R antibody is the Food and Drug Administration-approved monoclonal antibody tocilizumab, also known as atlizumab (marketed as Actemra, or RoActemra). Tocilizumab is a humanized monoclonal antibody against the interleukin-6 receptor (IL-6R). Administration of tocilizumab has demonstrated near-immediate reversal of CRS.

CRS is generally managed based on the severity of the observed syndrome and interventions are tailored as such. CRS management decisions may be based upon clinical signs and symptoms and response to interventions, not solely on laboratory values alone.

Mild to moderate cases generally are treated with symptom management with fluid therapy, non-steroidal anti-inflammatory drug (NSAID) and antihistamines as needed for adequate symptom relief. More severe cases include patients with any degree of hemodynamic instability; with any hemodynamic instability, the administration of tocilizumab is recommended. The first-line management of CRS may be tocilizumab, in some embodiments, at the labeled dose of 8 mg/kg IV over 60 minutes (not to exceed 800 mg/dose); tocilizumab can be repeated Q8 hours. If suboptimal response to the first dose of tocilizumab, additional doses of tocilizumab may be considered. Tocilizumab can be administered alone or in combination with corticosteroid

therapy. Patients with continued or progressive CRS symptoms, inadequate clinical improvement in 12-18 hours or poor response to tocilizumab, may be treated with high-dose corticosteroid therapy, generally hydrocortisone 100 mg IV or methylprednisolone 1-2 mg/kg. In patients with more severe hemodynamic instability or more severe respiratory symptoms, patients may be administered high-dose corticosteroid therapy early in the course of the CRS. CRS management guidance may be based on published standards (Lee et al. (2019) *Biol Blood Marrow Transplant*, doi.org/10.1016/j.bbmt.2018.12.758; Neelapu et al. (2018) *Nat Rev Clin Oncology*, 15:47; Teachey et al. (2016) *Cancer Discov*, 6 (6): 664-679).

Features consistent with Macrophage Activation Syndrome (MAS) or Hemophagocytic lymphohistiocytosis (HLH) have been observed in patients treated with CAR-T therapy (Henter, 2007), coincident with clinical manifestations of the CRS. MAS appears to be a reaction to immune activation that occurs from the CRS, and should therefore be considered a manifestation of CRS. MAS is similar to HLH (also a reaction to immune stimulation). The clinical syndrome of MAS is characterized by high grade non-remitting fever, cytopenias affecting at least two of three lineages, and hepatosplenomegaly. It is associated with high serum ferritin, soluble interleukin-2 receptor, and triglycerides, and a decrease of circulating natural killer (NK) activity.

In one aspect, the invention includes a method of treating cancer in a subject in need thereof, comprising administering to the subject any one of the modified immune or precursor cells disclosed herein. Yet another aspect of the invention includes a method of treating cancer in a subject in need thereof, comprising administering to the subject a modified immune or precursor cell generated by any one of the methods disclosed herein.

In one aspect, the invention includes a method for treating cancer in a subject in need thereof. The method comprises administering to the subject a modified T cell or precursor thereof comprising a bispecific CAR comprising a first antigen binding domain comprising a nanobody capable of binding CD13, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain.

In another embodiment, the method comprises administering to the subject a modified T cell or precursor thereof comprising a bispecific CAR comprising a first antigen binding domain comprising Nb157, a first intracellular domain comprising CD3 zeta, a second antigen binding domain capable of binding TIM-3, a CD28 transmembrane domain, and a second intracellular domain comprising 4-1BB.

In another aspect, the invention includes a method of treating cancer in a subject in need thereof comprising administering to the subject a modified T cell or precursor thereof comprising an inducible bispecific CAR comprising a first antigen binding domain capable of binding Peptide-Neo-Epitope (PNE), a first transmembrane domain, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain.

In another embodiment, the method comprises administering to the subject a modified T cell or precursor thereof comprising an inducible bispecific CAR comprising a first antigen binding domain capable of binding Peptide-Neo-Epitope (PNE), a first transmembrane domain, a first intracellular domain comprising CD3 zeta, a second antigen

binding domain capable of binding TIM-3, a CD28 transmembrane domain, and a second intracellular domain comprising 4-1BB.

The method may further comprise administering, along with the inducible bispecific CAR, a nanobody fused to PNE. In certain embodiments, the nanobody is specific for CD13. In certain embodiments, the nanobody is Nb157 and may comprise the amino acid sequence set forth in SEQ ID NO: 1.

10 In certain embodiments, the T cell is a human cell. In certain embodiments, the T cell is autologous. Vectors

A vector may be used to introduce the CAR into a T cell as described elsewhere herein. In certain aspects, the invention includes vectors comprising nucleic acid sequences encoding a CAR. The vector can comprise a plasmid vector, viral vector, retrotransposon (e.g. piggyback, sleeping beauty), site directed insertion vector (e.g. CRISPR, Zn finger nucleases, TALEN), suicide expression vector, lentiviral vector, RNA vector, or other known vector in the art.

15 The production of any of the molecules described herein can be verified by sequencing. Expression of the full length proteins may be verified using immunoblot, immunohistochemistry, flow cytometry or other technology well known and available in the art.

20 The present invention also provides a vector in which DNA of the present invention is inserted. Vectors, including those derived from retroviruses such as lentivirus, are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses, such as murine leukemia viruses, in that they can transduce non-proliferating cells, such as hepatocytes. They 25 also have the added advantage of resulting in low immunogenicity in the subject into which they are introduced.

25 The expression of natural or synthetic nucleic acids is typically achieved by operably linking a nucleic acid or portions thereof to a promoter, and incorporating the construct into an expression vector. The vector is one generally capable of replication in a mammalian cell, and/or also capable of integration into the cellular genome of the mammal. Typical vectors contain transcription and translation terminators, initiation sequences, and promoters useful 30 for regulation of the expression of the desired nucleic acid sequence.

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

40 The expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al., 2012, MOLECULAR CLONING: A LABORATORY MANUAL, volumes 1-4, Cold Spring Harbor Press, NY), and in other virology and molecular biology manuals.

45 Viruses, which are useful as 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).

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.

An example of a promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, the EF-1 alpha 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 order to assess expression of a 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 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.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assessed 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 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.

#### Introduction of Nucleic Acids

Methods of introducing nucleic acids into a cell include physical, biological and chemical methods. Physical methods for introducing a polynucleotide, such as RNA, into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. RNA can be introduced into target cells using commercially available methods which include 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). RNA can also be introduced into cells using cationic liposome mediated transfection using lipofection, using polymer encapsulation, using peptide mediated transfection, or using biolistic particle delivery systems such as "gene guns" (see, for example, Nishikawa, et al. Hum Gene Ther., 12 (8): 861-70 (2001)).

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).

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 nucleic acids 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.

Moreover, the nucleic acids may be introduced by any means, such as transducing the expanded T cells, transfecting the expanded T cells, and electroporating the expanded T cells. One nucleic acid may be introduced by one method and another nucleic acid may be introduced into the T cell by a different method.

#### RNA

In one embodiment, the nucleic acids introduced into the T cell are RNA. In another embodiment, the RNA is mRNA that comprises in vitro transcribed RNA or synthetic RNA. The RNA is produced by in vitro transcription using a polymerase chain reaction (PCR)-generated template. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. The desired template for in vitro transcription is a chimeric membrane protein. By way of example, the template encodes an antibody, a fragment of an antibody or a portion of an antibody. By way of another example, the template comprises an extracellular domain comprising a single chain variable domain of an antibody, such as anti-CD3, and an intracellular domain of a co-stimulatory molecule. In one embodiment, the template for the RNA chimeric membrane protein encodes a chimeric membrane protein comprising an extracellular domain comprising an antigen binding domain derived from an antibody to a co-stimulatory molecule, and an intracellular domain derived from a portion of an intracellular domain of CD28 and 4-1BB.

PCR can be used to generate a template for in vitro transcription of mRNA which is then introduced into cells. Methods for performing PCR are well known in the art. Primers for use in PCR are designed to have regions that are substantially complementary to regions of the DNA to be used as a template for the PCR. "Substantially complementary", as used herein, refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are complementary, or one or more bases are non-complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of a gene that is normally transcribed in cells (the open reading frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a gene that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR are generated by synthetic methods that are well known in the art. "Forward primers" are primers that contain a region of nucleotides that are substantially complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. "Upstream" is used herein to refer to a location 5, to the

DNA sequence to be amplified relative to the coding strand. "Reverse primers" are primers that contain a region of nucleotides that are substantially complementary to a double-stranded DNA template that are downstream of the DNA sequence that is to be amplified. "Downstream" is used herein to refer to a location 3' to the DNA sequence to be amplified relative to the coding strand.

Chemical structures that have the ability to promote stability and/or translation efficiency of the RNA may also be used. The RNA preferably has 5' and 3' UTRs. In one embodiment, the 5' UTR is between zero and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to, designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the gene of interest. Alternatively, UTR sequences that are not endogenous to the gene of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the gene of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of mRNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art.

In one embodiment, the 5' UTR can contain the Kozak sequence of the endogenous gene. Alternatively, when a 5' UTR that is not endogenous to the gene of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many mRNAs is known in the art. In other embodiments the 5' UTR can be derived from an RNA virus whose RNA genome is stable in cells. In other embodiments various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the mRNA.

To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In one embodiment, the promoter is a T7 polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters. Consensus nucleotide sequences for T7, T3 and SP6 promoters are known in the art.

In one embodiment, the mRNA has both a cap on the 5' end and a 3' poly(A) tail which determine ribosome binding, initiation of translation and stability mRNA in the cell. On a circular DNA template, for instance, plasmid DNA, RNA polymerase produces a long concatameric product which is not suitable for expression in eukaryotic cells. The transcription of plasmid DNA linearized at the end of the 3' UTR results in normal sized mRNA which is not effective in eukaryotic transfection even if it is polyadenylated after transcription.

On a linear DNA template, phage T7 RNA polymerase can extend the 3' end of the transcript beyond the last base of the template (Schenborn and Mierendorf, Nuc Acids Res., 13:6223-36 (1985); Nacheva and Berzal-Herranz, Eur. J. Biochem., 270:1485-65 (2003).

The conventional method of integration of polyA/T stretches into a DNA template is molecular cloning. However polyA/T sequence integrated into plasmid DNA can cause plasmid instability, which is why plasmid DNA templates obtained from bacterial cells are often highly contaminated with deletions and other aberrations. This makes cloning procedures not only laborious and time consuming but often not reliable. That is why a method which allows construction of DNA templates with polyA/T 3' stretch without cloning highly desirable.

The polyA/T segment of the transcriptional DNA template can be produced during PCR by using a reverse primer containing a polyT tail, such as 100T tail (size can be 50-5000 T), or after PCR by any other method, including, but not limited to, DNA ligation or in vitro recombination. Poly(A) tails also provide stability to RNAs and reduce their degradation. Generally, the length of a poly(A) tail positively correlates with the stability of the transcribed RNA. In one embodiment, the poly(A) tail is between 100 and 5000 adenosines.

Poly(A) tails of RNAs can be further extended following in vitro transcription with the use of a poly(A) polymerase, such as *E. coli* polyA polymerase (E-PAP). In one embodiment, increasing the length of a poly(A) tail from 100 nucleotides to between 300 and 400 nucleotides results in about a two-fold increase in the translation efficiency of the RNA. Additionally, the attachment of different chemical groups to the 3' end can increase mRNA stability. Such attachment can contain modified/artificial nucleotides, aptamers and other compounds. For example, ATP analogs can be incorporated into the poly(A) tail using poly(A) polymerase. ATP analogs can further increase the stability of the RNA.

5' caps also provide stability to RNA molecules. In a preferred embodiment, RNAs produced by the methods disclosed herein include a 5' cap. The 5' cap is provided using techniques known in the art and described herein (Cougot, et al., Trends in Biochem. Sci., 29:436-444 (2001); Stepinski, et al., RNA, 7:1468-95 (2001); Elango, et al., Biochim. Biophys. Res. Commun., 330:958-966 (2005)).

The RNAs produced by the methods disclosed herein can also contain an internal ribosome entry site (IRES) sequence. The IRES sequence may be any viral, chromosomal or artificially designed sequence which initiates cap-independent ribosome binding to mRNA and facilitates the initiation of translation. Any solutes suitable for cell electroporation, which can contain factors facilitating cellular permeability and viability such as sugars, peptides, lipids, proteins, antioxidants, and surfactants can be included.

In some embodiments, the RNA is electroporated into the cells, such as in vitro transcribed RNA.

The disclosed methods can be applied to the modulation of T 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 to kill a target cancer cell.

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 RNA, making it possible to individually regulate the expression level. Furthermore, the PCR-based technique of mRNA production

greatly facilitates the design of the mRNAs with different structures and combination of their domains.

One advantage of RNA transfection methods of the invention is that RNA transfection is essentially transient and a vector-free. A RNA transgene can be delivered to a lymphocyte and expressed therein following a brief in vitro cell activation, as a minimal expressing cassette without the need for any additional viral sequences. Under these conditions, integration of the transgene into the host cell genome is unlikely. Cloning of cells is not necessary because of the efficiency of transfection of the RNA and its ability to uniformly modify the entire lymphocyte population.

Genetic modification of T cells with in vitro-transcribed RNA (IVT-RNA) makes use of two different strategies both of which have been successively tested in various animal models. Cells are transfected with in vitro-transcribed RNA by means of lipofection or electroporation. It is desirable to stabilize IVT-RNA using various modifications in order to achieve prolonged expression of transferred IVT-RNA.

Some IVT vectors are known in the literature which are utilized in a standardized manner as template for in vitro transcription and which have been genetically modified in such a way that stabilized RNA transcripts are produced. Currently protocols used in the art are based on a plasmid vector with the following structure: a 5' RNA polymerase promoter enabling RNA transcription, followed by a gene of interest which is flanked either 3' and/or 5' by untranslated regions (UTR), and a 3' polyadenyl cassette containing 50-70 A nucleotides. Prior to in vitro transcription, the circular plasmid is linearized downstream of the polyadenyl cassette by type II restriction enzymes (recognition sequence corresponds to cleavage site). The polyadenyl cassette thus corresponds to the later poly(A) sequence in the transcript. As a result of this procedure, some nucleotides remain as part of the enzyme cleavage site after linearization and extend or mask the poly(A) sequence at the 3' end. It is not clear, whether this nonphysiological overhang affects the amount of protein produced intracellularly from such a construct.

RNA has several advantages over more traditional plasmid or viral approaches. Gene expression from an RNA source does not require transcription and the protein product is produced rapidly after the transfection. Further, since the RNA has to only gain access to the cytoplasm, rather than the nucleus, and therefore typical transfection methods result in an extremely high rate of transfection. In addition, plasmid based approaches require that the promoter driving the expression of the gene of interest be active in the cells under study.

In another aspect, the RNA construct is delivered into the cells by electroporation. See, e.g., the formulations and methodology of electroporation of nucleic acid constructs into mammalian cells as taught in US 2004/0014645, US 2005/0052630A1, US 2005/0070841A1, US 2004/0059285A1, US 2004/0092907A1. The various parameters including electric field strength required for electroporation of any known cell type are generally known in the relevant research literature as well as numerous patents and applications in the field. See e.g., U.S. Pat. Nos. 6,678,556, 7,171,264, and 7,173,116. Apparatus for therapeutic application of electroporation are available commercially, e.g., the MedPulser™ DNA Electroporation Therapy System (Inovio/Genetronics, San Diego, Calif.), and are described in patents such as U.S. Pat. Nos. 6,567,694; 6,516,223, 5,993,434, 6,181,964, 6,241,701, and 6,233,482; electroporation may also be used for transfection of cells in vitro as described e.g. in US20070128708A1. Electroporation may

also be utilized to deliver nucleic acids into cells in vitro. Accordingly, electroporation-mediated administration into cells of nucleic acids including expression constructs utilizing any of the many available devices and electroporation systems known to those of skill in the art presents an exciting new means for delivering an RNA of interest to a target cell.

#### Sources of T Cells

In certain embodiments, a source of T cells is obtained from a subject. Non-limiting examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Preferably, the subject is a human. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, spleen tissue, umbilical cord, and tumors. In certain embodiments, any number of T cell lines available in the art, may be used. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll separation. In one embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. The cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media, such as phosphate buffered saline (PBS) or wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations, for subsequent processing steps. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

In another embodiment, T cells are isolated from peripheral blood by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PER-COLL™ gradient. Alternatively, T cells can be isolated from umbilical cord. In any event, a specific subpopulation of T cells can be further isolated by positive or negative selection techniques.

The cord blood mononuclear cells so isolated can be depleted of cells expressing certain antigens, including, but not limited to, CD34, CD8, CD14, CD19 and CD56. Depletion of these cells can be accomplished using an isolated antibody, a biological sample comprising an antibody, such as ascites, an antibody bound to a physical support, and a cell bound antibody.

Enrichment of a T cell population by negative selection can be accomplished using a combination of antibodies directed to surface markers unique to the negatively selected cells. A preferred 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.

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 one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, 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.

T cells can also be frozen after the washing step, which does not require the monocyte-removal step. While not wishing 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, in a non-limiting example, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or other suitable cell freezing media. The cells are then 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 one embodiment, the population of T cells is comprised within cells such as peripheral blood mononuclear cells, cord blood cells, a purified population of T cells, and a T cell line. In another embodiment, peripheral blood mononuclear cells comprise the population of T cells. In yet another embodiment, purified T cells comprise the population of T cells.

In certain embodiments, T regulatory cells (Tregs) can be isolated from a sample. The sample can include, but is not limited to, umbilical cord blood or peripheral blood. In certain embodiments, the Tregs are isolated by flow-cytometry sorting. The sample can be enriched for Tregs prior to isolation by any means known in the art. The isolated Tregs can be cryopreserved, and/or expanded prior to use. Methods for isolating Tregs are described in U.S. Pat. Nos. 7,754,482, 8,722,400, and 9,555,105, and U.S. patent application Ser. No. 13/639,927, contents of which are incorporated herein in their entirety.

#### Expansion of T Cells

In certain embodiments, the T cells disclosed herein can be multiplied by about 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold, 100 fold, 200 fold, 300 fold, 400 fold, 500 fold, 600 fold, 700 fold, 800 fold, 900 fold, 1000 fold, 2000 fold, 3000 fold, 4000 fold, 5000 fold, 6000 fold, 7000 fold, 8000 fold, 9000 fold, 10,000 fold, 100,000 fold, 1,000,000 fold, 10,000,000 fold, or greater, and any and all whole or partial integers therebetween. In one embodiment, the T cells expand in the range of about 20 fold to about 50 fold.

Following culturing, the T cells can be incubated in cell medium in a culture apparatus for a period of time or until the cells reach confluence or high cell density for optimal passage before passing the cells to another culture apparatus. The culturing apparatus can be of any culture apparatus commonly used for culturing cells in vitro. Preferably, the level of confluence is 70% or greater before passing the cells to another culture apparatus. More preferably, the level of confluence is 90% or greater. A period of time can be any time suitable for the culture of cells in vitro. The T cell medium may be replaced during the culture of the T cells at any time. Preferably, the T cell medium is replaced about every 2 to 3 days. The T cells are then harvested from the culture apparatus whereupon the T cells can be used imme-

dately or cryopreserved to be stored for use at a later time. In one embodiment, the invention includes cryopreserving the expanded T cells. The cryopreserved T cells are thawed prior to introducing nucleic acids into the T cell.

In another embodiment, the method comprises isolating T cells and expanding the T cells. In another embodiment, the invention further comprises cryopreserving the T cells prior to expansion. In yet another embodiment, the cryopreserved T cells are thawed for electroporation with the RNA encoding the chimeric membrane protein.

Another procedure for ex vivo expansion cells is described in U.S. Pat. No. 5,199,942 (incorporated herein by reference). Expansion, such as described in U.S. Pat. No. 5,199,942 can be an alternative or in addition to other methods of expansion described herein. Briefly, ex vivo culture and expansion of T cells comprises the addition to the cellular growth factors, such as those described in U.S. Pat. No. 5,199,942, or other factors, such as flt3-L, IL-1, IL-3 and c-kit ligand. In one embodiment, expanding the T cells comprises culturing the T cells with a factor selected from the group consisting of flt3-L, IL-1, IL-3 and c-kit ligand.

The culturing step as described herein (contact with agents as described herein or after electroporation) can be very short, for example less than 24 hours such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 hours. The culturing step as described further herein (contact with agents as described herein) can be longer, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or more days.

Various terms are used to describe cells in culture. Cell culture refers generally to cells taken from a living organism and grown under controlled condition. A primary cell culture is a culture of cells, tissues or organs taken directly from an organism and before the first subculture. Cells are expanded in culture when they are placed in a growth medium under conditions that facilitate cell growth and/or division, resulting in a larger population of the cells. When cells are expanded in culture, the rate of cell proliferation is typically measured by the amount of time required for the cells to double in number, otherwise known as the doubling time.

Each round of subculturing is referred to as a passage. When cells are subcultured, they are referred to as having been passaged. A specific population of cells, or a cell line, is sometimes referred to or characterized by the number of times it has been passaged. For example, a cultured cell population that has been passaged ten times may be referred to as a P10 culture. The primary culture, i.e., the first culture following the isolation of cells from tissue, is designated P0. Following the first subculture, the cells are described as a secondary culture (P1 or passage 1). After the second subculture, the cells become a tertiary culture (P2 or passage 2), and so on. It will be understood by those of skill in the art that there may be many population doublings during the period of passaging; therefore the number of population doublings of a culture is greater than the passage number. The expansion of cells (i.e., the number of population doublings) during the period between passaging depends on many factors, including but is not limited to the seeding density, substrate, medium, and time between passaging.

In one embodiment, the cells may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. 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), inter-

leukin-2 (IL-2), insulin, IFN-gamma, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF-beta, and TNF-alpha, 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, alpha-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or 10 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 15 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% CO<sub>2</sub>).

The medium used to culture the T cells may include an 20 agent that can co-stimulate the T cells. For example, an agent that can stimulate CD3 is an antibody to CD3, and an agent that can stimulate CD28 is an antibody to CD28. This is because, as demonstrated by the data disclosed herein, a cell isolated by the methods disclosed herein can be 25 expanded approximately 10 fold, 20 fold, 30 fold, 40 fold, 50 fold, 60 fold, 70 fold, 80 fold, 90 fold, 100 fold, 200 fold, 300 fold, 400 fold, 500 fold, 600 fold, 700 fold, 800 fold, 900 fold, 1000 fold, 2000 fold, 3000 fold, 4000 fold, 5000 fold, 6000 fold, 7000 fold, 8000 fold, 9000 fold, 10,000 fold, 30 100,000 fold, 1,000,000 fold, 10,000,000 fold, or greater. In one embodiment, the T cells expand in the range of about 20 fold to about 50 fold, or more by culturing the electroporated population. In one embodiment, human T regulatory cells are expanded via anti-CD3 antibody coated KT64.86 artificial antigen presenting cells (aAPCs). Methods for expanding and activating T cells can be found in U.S. Pat. Nos. 7,754,482, 8,722,400, and 9,555,105, contents of which are incorporated herein in their entirety.

In one embodiment, the method of expanding the T cells 40 can further comprise isolating the expanded T cells for further applications. In another embodiment, the method of expanding can further comprise a subsequent electroporation of the expanded T cells followed by culturing. The subsequent electroporation may include introducing a nucleic acid encoding an agent, such as a transducing the expanded T cells, transfecting the expanded T cells, or electroporating the expanded T cells with a nucleic acid, into the expanded population of T cells, wherein the agent further stimulates the T cell. The agent may stimulate the T cells, 45 such as by stimulating further expansion, effector function, or another T cell function.

#### Pharmaceutical Compositions

Pharmaceutical compositions of the present invention 50 may comprise the modified T cell 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; 55 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.

60 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

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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.

The cells of the invention to be administered may be autologous, allogeneic or xenogeneic with respect to the subject undergoing therapy.

Cells of the invention can be administered in dosages and routes and at times to be determined in appropriate pre-clinical and clinical experimentation and trials. Cell compositions may be administered multiple times at dosages within these ranges. Administration of the cells of the invention may be combined with other methods useful to treat the desired disease or condition as determined by those of skill in the art.

It can generally be stated that a pharmaceutical composition comprising the modified T cells described herein may be administered at a dosage of  $10^4$  to  $10^9$  cells/kg body weight, in some instances  $10^5$  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.

The administration of the modified T cells of the invention may be carried out in any convenient manner known to those of skill in the art. The cells of the present invention may be administered to a subject by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient transarterially, subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. In other instances, the cells of the invention are injected directly into a site of inflammation in the subject, a local disease site in the subject, a lymph node, an organ, a tumor, and the like.

It should be understood that the method and compositions that would be useful in the present invention are not limited to the particular formulations set forth in the examples. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the cells, expansion and culture methods, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", fourth edition (Sambrook, 2012); "Oligonucleotide Synthesis" (Gait, 1984); "Culture of Animal Cells" (Freshney, 2010); "Methods in Enzymology" "Handbook of Experimental Immunology" (Weir, 1997); "Gene Transfer Vectors for Mammalian Cells" (Miller and Calos, 1987); "Short Protocols in Molecular Biology" (Ausubel, 2002); "Current Protocols in Immunology" (Coligan, 2002). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and

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practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

## EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

The Materials and Methods used in the performance of the experiments disclosed herein are now described.

Nb phage library construction from THP-1 cell-immunized llama: A llama was immunized with  $2 \times 10^7$  THP-1 cells (Caprolitics, Hardwick, MA) once a month for 3 months. Peripheral blood mononuclear cell isolation, RNA extraction, and complementary DNA (cDNA) synthesis were performed as previously described (Zhang et al., *J Immunol.* 2016; 196 (4): 1591-1603).

Animals and in vivo models: All laboratory mice were maintained on a 12 hr light-dark cycle. NOD/Shi-scid/IL-2R<sup>y</sup>null (NSG) mice, 8-12 weeks old, were obtained from Jackson Laboratories. NSG mice were inoculated with  $1 \times 10^7$  cells of THP-1 or HL60 subcutaneously, or with  $0.5 \times 10^7$  cells of K562 subcutaneously. When tumor volume reached  $100 \text{ mm}^3$  about 12 days after xenograft, Nb CAR T cells or untransduced (UTD) human T cells ( $1 \times 10^7$  cells) were administrated via tail vein. Mice and tumors were monitored every other day. Tumor dimensions were measured with Vernier calipers and tumor volume was calculated as  $\frac{1}{2}$  larger diameter  $\times$  (smaller diameter).

NSG mice were conditioned by Busulfex (30 mg/kg) 24 hrs prior to tail injection with  $2 \times 10^7$  of patient-derived AML cells. Two weeks later CAR or UTD T cells were transduced into the mice. The recipient mice were sacrificed at the experimental end point based on the protocol, and the long bones (femurs), spleens and livers were collected for histological analysis by H & E staining. Mice were sacrificed according to protocol when moribund or upon the development of hind-limb paralysis.

Statistical analysis: Microsoft Excel and GraphPad Prism software was used for statistical analysis. Student's t test was used to determine the significance of the results unless otherwise indicated. Kaplan-Meier statistical analysis was performed using the log rank test. In the figures, asterisks denote statistically significant p values (\*, p<0.05, \*\*, p<0.01, \*\*\*, p<0.001), and "ns" indicates lack of statistical significance (p>0.05).

Cell Lines, Cell Culture, Plasmids and Antibodies: The THP-1, Jurkat, K562, HL60, U937, MV4-11, NB4 and SKOV3 cell lines were obtained from the ATCC and maintained in RPMI1640 with 10% FBS and 1% penicillin/streptomycin (R10 medium) and maintained at 37° C. and 5% CO<sub>2</sub>. HEK293T cells were obtained from ATCC and cultured in DMEM supplemented with 10% fetal bovine

serum (FBS). NET NT-3 cell line was cultured in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, HEPES, EGF (20 ng/mL), and FGF2 (10 ng/mL). Deidentified Patient derived AML cells were obtained from the University of Pennsylvania Stem Cell and Xenograft Core facility, and maintained in the R10 medium. Normal donor total T cells were obtained from the Human Immunology Core at University of Pennsylvania, and maintained in the R10 medium.

pComb3XSS was a gift from Carlos Barbas (Addgene plasmid #63890). pHIV-EGFP was a gift from Bryan Welm & Zena Werb (Addgene plasmid #21373). lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid #52961). Human membrane protein cDNA library were provided by High-throughput Screen Core at University of Pennsylvania.

In vitro analysis of T cell function: For T-cell transduction, HEK293T cells were co-transfected with lenti-vector plasmid, psPAX2 and VSV-G plasmid DNA to produce the lentivirus 48 h after transfection. Normal donor T cells were positively selected from leukapheresis packs using anti-CD4 and CD8 microbeads (Miltenyi), expanded in vitro with anti-CD3/CD28 beads (Invitrogen) for up to 12 days. Total T cells were transduced with lentivirus 24 hours after activation. The resulting virus from the supernatant were concentrated via ultracentrifugation at 25,000 g for 2.5 h at 4° C.

Killing assays were performed as previously described (Cao, L. F. et al. (2010) Cytometry A 77, 534-545). In brief, target cells were labeled by anti-CD33 (BD) for detecting cell number with flow cytometry analysis or labeled by CellTrace Far Red for tracing cell division. Target cells were incubated with effector T cells for 16 hrs at a series of ratios. Cells were then harvested, washed, and stained by Propidium Iodide prior to flow cytometry analysis. Quantification was calculated by either Countbright beads or volume.

To detect cytokine secretion, effector and target cells were incubated at a 1:1 ratio in R10 medium for 16 hrs as indicated. Supernatant was analyzed using Human TNF-alpha or IFN-gamma DuoSet ELISA kits according to the manufacturer's instructions (R&D System).

To detect cell degranulation, activated and Nb CAR transduced or untransduced T cells ( $1 \times 10^5$  cells) were cocultured with THP-1 or K526 cells at a 1:1 ratio in 96-well plates for 4 hrs, in the presence of APC-conjugated anti-CD107a antibody, followed by washing and flow cytometry analysis.

To monitor cell proliferation assay, T cells were labeled by CellTrace™ Far Red Cell Proliferation Kit (Invitrogen) according to the manufacturer's instructions. The reaction was quenched with R10 medium, and the cells were washed twice. T cells were incubated at a 1:1 ratio with heat-inactivated target cells for 96 hrs.

Switchable CAR (sCAR) T system: Anti-PNE single chain variable fragment (scFv) (Zahnd C, et al. (2004) *J Biol Chem.* April 30; 279 (18): 18870-7) was custom synthesized by GeneArt (Rodgers, D. T. et al. (2016) *Proceedings of the National Academy of Sciences of the United States of America* 113, E459-468), followed by insertion into pHIV-41BB-CD3z vector. sCAR lentivirus was packaged and used to transduce human T cells. sCAR expression was detected by flow cytometry and western blot. Nb157 with C-terminal PNE (Nb157-C-PNE) or with N-terminal PNE (Nb157-N-PNE) were constructed by molecular cloning, followed by prokaryotic expression and purification via Ni-NTA affinity (QIAGEN) in TOP10 (Invitrogen) induced by isopropyl-β-d-thiogalactoside (IPTG).

The Results the experiments disclosed herein are now described.

#### Example 1: Anti-TIM-3 CAR

An anti-TIM-3 CAR comprised of an anti-TIM-3-scFv, an IgG4 hinge, a CD28 transmembrane domain, and 4-1BB and CD3z intracellular domains, was generated herein (FIG. 1C). TIM-3 was expressed on NB4-TIM-3 cell lines, as measured by Western Blot (FIG. 1A). NB4 or NB4-TIM-3 cells were incubated with anti-TIM-3-scFv-CAR T cells overnight and cytotoxicity was measured by flow cytometry analysis (FIG. 1B). Untransduced (UTD) T cells were used as a negative control. The data showed that anti-TIM3 CAR T cells specifically killed TIM-3-expressing myeloid leukemia cells. (FIG. 1D).

#### Example 2: Bispecific Split TIM-3&CD13 CAR

A bispecific split CAR was generated herein that specifically kills tumor cells expressing TIM-3 and CD13 (FIG. 2). The CAR was comprised of a nanobody specific for CD13 (Nb157), a CD3zeta domain, a P2A linker, an anti-TIM3-scFv, a CD28 transmembrane domain, and a 4-1BB intracellular domain. Bispecific split CAR T cells targeting CD13 and TIM-3 or control UTD T cells were injected into NSG mice transplanted with either NB4 cells or NB4-TIM3, and monitored for tumor growth. The results demonstrated that the bispecific split CAR (BissCAR) T cells eradicated the NB4-TIM3 tumors expressing both CD13 and TIM-3, but not the NB4 tumors that only express CD13.

#### Example 3: Inducible, Bispecific TIM-3&CD13 CAR

An inducible bispecific CAR was generated herein that specifically kills AML leukemic stem cells (LSCs) that express both TIM-3 and CD13 (FIG. 4B). Two CARs were linked by the P2A construct. The first CAR comprised an anti-TIM-3-scFv, a CD28 hinge, a CD28 transmembrane domain, and CD28 and 4-1BB intracellular domains. The second, switchable CAR (sCAR) was comprised of an anti-PNE-scFv, an IgG4 hinge, a CD8 transmembrane domain, and a CD3zeta intracellular domain (FIG. 3A). T cells expressing the bispecific CAR were incubated with human AML LSCs, which expressed both TIM-3 and CD13 (FIG. 3B). In the absence of Nb157-PNE, the sCAR bound the LSC by TIM-3 recognition, but didn't exhibit cytotoxicity (FIG. 3C). In the presence of the Nb157-PNE switch, the T cell fully activated and killed the LSC targets (FIG. 3C).

#### Example 4: Bispecific CARs of Nb157 and Anti-TIM3 Eradicate AML In Vivo in a Safer Manner

CAR T therapy may cause serious cytokine release syndrome and other on-target/off-tumor side effects. To reduce this risk, as well as further attenuate the potential side effects of targeting moderately expressed CD13 in non-AML cells with low CD13 expression, such as hematopoietic stem cells, a logic-gated and controllable system to manipulate the antitumor activity of Nb157 CAR T cells to AML was developed. TIM3, an immune suppressing receptor, is highly expressed on the majority of human AML leukemia stem cells (LSCs), but not on human HSCs. To overcome the challenge that Nb157 CAR T cells kill both AML cells and

HSCs, a combinational bispecific CAR T system was designed to specifically kill AML LSC that commonly express both TIM3 and CD13 (FIG. 4A), which effectively kills CD13+TIM3+ LSC cells, yet spares normal cells that only have CD13 expression.

To prove that the bispecific CAR T cells could eradicate LSCs but spare HSCs, a NB4 and NB4-TIM3 cell model was first constructed, which mimicked HSCs (CD13+ TIM3-) and LSCs (CD13+TIM3+) (FIG. 1B). A TIM3 CAR T cell was constructed with a published anti-TIM3 antibody (U.S. Pat. No. 9,605,070B2), followed by a linker, a transmembrane domain (TM), 4-1BB domain, and CD3 zeta domain (FIG. 1C). An *in vitro* killing experiment showed that the TIM3 CAR T cell exhibited potent and specific cytotoxicity against NB4-TIM3 cells, but ignored the NB4 cells as UTD T cells (FIG. 1D).

An Nb157&TIM3 combinational bispecific CAR was generated (FIGS. 3A and 4A), wherein the Nb157 recognizes CD13 activated CD3z signaling, and the antiTIM3scFv recognizes TIM3 transduced CD28 and 4-1BB co-stimulatory signaling. To overcome the challenge that Nb157 CAR T cells may kill both AML and HSCs, the Nb157&TIM3 CAR was transduced into human primary T cells by lentivirus, and the resulting T cells were used for flow cytometry to detect expression of both Nb157 (using a rabbit anti-llama VHH) and TIM3 (using an anti-mouse scFv). The results showed that both CARs were co-expressed on the surface of the transduced CAR T cells simultaneously (FIG. 3B). An *in vitro* killing assays showed Nb157&TIM3 CAR T cells showed potent toxicity against both NB4 and NB4-TIM3 cells. The CD3zeta cytotoxicity signaling was elicited by CD13 recognition, which is commonly expressed on leukemia cells (FIG. 3C). In the *in vivo* tumor killing assay, Nb157&TIM3 CAR T cells suppressed NB4 tumor growth (FIG. 5A), but eradicated the NB4-TIM3 tumor (FIG. 5B), which confirmed that the combination of bispecific and split CAR was needed to completely regress the tumor while sparing the single-antigen-expressing normal cells. Meanwhile, the Nb157&TIM3 CAR T cells in the NB4-TIM3 tumor mice proliferated one-fold more than in the NB4 tumor mice (FIG. 6), which also supported that *in vivo* tumor eradication requires sustainability and enhanced activity of the T cells.

The combinational bispecific CAR system was tested to determine if it could suppress primary AML cells from patients. Patient-derived AML cells were injected into NSG mice to induce leukemia, as determined by detection of the human CD33+ AML cells in the peripheral blood of the recipient mice (FIG. 7A). The leukemic mice were treated with Nb157&TIM3 CAR or UTD T cells two weeks after leukemia injection (FIG. 7A). The appearance of CD33+ AML cells or CD3+ T cells in peripheral blood were monitored by flow cytometry analysis weekly (FIGS. 7A-7C). The results indicated that peripheral blood AML cells, following the first week of injection, gradually decreased in the Nb157&TIM3 CAR T group (FIG. 7B), consistent with heavy leukemia infiltration in the spleen in later stage. Notably, treatment with Nb157&TIM3 CAR, but not UTD T cells, increased the number of peripheral T cells one week after the T cell injection, reflecting the quick activation and proliferation of the CAR T cells to kill the AML cells (FIG. 7C). Consistent with this observation, Kaplan Maier analysis showed that treatment with Nb157&TIM3 CAR T cells significantly prolonged survival compared to mice treated with UTD T cells (FIG. 7D). Therefore, the results demonstrated that the Nb157&TIM3

CAR T cells targeting both CD13 and TIM3 can effectively eradicate the double-positive patient-derived AML cells in a clinically relevant model.

The impact of Nb157&TIM3 CAR T cells on normal hematopoiesis was investigated. Humanized immune system mice (HIS mice) were employed to assess hematopoietic toxicity of Nb157&TIM3 CAR T cells (FIG. 7E). NSG mice were conditioned by busulfan and engrafted with bone marrow CD34+ cells from normal adult donors, followed by treatment with Nb157&TIM3 CAR, Nb157 CAR, or UTD T cells four weeks later. Mice were bled before and after T cell treatment to confirm the engraftment and analyze the peripheral constitution of the mice. Bone marrow from the mice was collected for analysis three weeks after treatment (FIG. 7E). Based on CD13 expression on normal HSCs, analysis of the bone marrow three weeks after treatment showed near disappearance of both CD34+CD38- hematopoietic stem cells and CD34+CD38+ myeloid progenitors in the conventional Nb157 CAR group (FIGS. 87F-7H). However, the Nb157&TIM3 bispecific CARs showed ameliorated disruption in normal HSCs (FIGS. 7E-7F). Therefore, these results demonstrated that the Nb157&TIM3 CAR T cells effectively and safely eradicated patient-derived AML cells in the clinically relevant model.

#### Example 5: Combinatory Bispecific and Split CAR T Cells Targeting CD13 and TIM3 Redirect T Cells to Eradicate AML Xenografts and AML PDXs *In Vivo*

Because CAR T-cell therapy may cause on-target/off-tumor side effects, it is ideal to reduce the toxicity by increasing the specificity with multiple tumor markers. In this regard, novel bispecific CAR T cells were developed to synergistically kill the experimental tumor models by targeting >1 tumor-associated antigen (TAA).

One other potential TAA, TIM3, an immune-suppressing receptor, is highly expressed in the majority of human AML LSCs, but not in HSCs. A combinatory bispecific and split CAR (BissCAR) T-cell system was developed to effectively kill CD13+TIM3+ LSCs, while maintaining a reduced impact on normal cells that only express CD13 (FIG. 4A). TIM3 expression was extremely low in normal donor bone marrow but high in the LSC subset (CD34+CD38-CD90-) (FIG. 4B, upper panels). In contrast, a high percentage of TIM3 and CD13 double-positive cells was detected in the LSC-enriched population (CD34+CD38-) from PD AML cells but not normal donor bone marrow (FIG. 4B), indicating the high coexpression of CD13 and TIM3 in LSGs.

NB4 (CD13+TIM3-) and NB4-TIM3 (CD13+TIM3+) cell lines were generated to mimic the HSG and LSC models (FIG. 1A-1B). Next, a conventional TIM3-BBz CAR was generated (FIG. 1C), which guided the T cells to kill NB4-TIM3 cells potently and specifically *in vitro* and suppressed NB4-TIM3 tumor growth *in vivo* (FIG. 1D).

Next, the BissCAR was constructed, in which Nb157 recognizing CD13 was linked to CD3z and anti-TIMS scFv recognizing TIM3 was linked to CD28 and 4-1BB costimulatory domains (FIG. 2; FIG. 4A). The resulting BissCAR expression on the T cells was verified by flow cytometry (FIG. 3B). An *in vitro* killing assay showed that BissCAR T cells killed NB4 and NB4-TIM3 cells, because the CD13 recognition elicited CD3z signaling to induce target death *in vitro* (FIG. 3C).

In the NB4 xenograft models, BissCAR T cells only moderately suppressed tumor growth compared with complete elimination when using Nb157 CAR T-cell treatment

(FIG. 5A). However, BissCAR T cells could eradicate the NB4-TIM3 tumor as potently as Nb157 CAR T cells (FIG. 5B). These results indicate that BissCAR T cells are capable of completely shrinking the tumor expressing GDIS and TIMS, but they spared the cells expressing only CD13. Consistently, BissCAR T-cell number in peripheral blood in NB4-TIM3 tumor-bearing mice was significantly higher than in NB4 tumor-bearing mice (FIG. 6).

It was explored whether BissCAR T cells could suppress RD AML cells. To this end, RD AML cells were transplanted into NSG mice to induce leukemia, followed by treatment with BissCAR or UTD T cells 2 weeks later (FIG. 7A). The appearance of CD33<sup>+</sup> AML cells or CD3<sup>+</sup> T cells in peripheral blood was monitored weekly (FIGS. 7B-7C). The results indicated that, following the first week of injection, peripheral blood AML cells gradually decreased in the BissCAR T-cell group (FIG. 7B), consistent with heavy leukemic infiltration in the spleen in the later stage. Notably, treatment with BissCAR T cells, but not with UTD T cells, increased peripheral T-cell number 1 week after the T-cell injection, reflecting the quick activation and proliferation of CAR T cells to kill AML cells (FIG. 7C). Consistently, BissCAR T-cell treatment significantly prolonged survival of the mice compared with the UTD T-cell group (FIG. 7D). It has been reported that various immune-suppressing factors weaken the immunotherapy for AML, such as the PD-1, TIM3 immune checkpoint molecules, and regulatory T cells (Tregs). BissCAR T cells and UTD T cells have similar low PD-1 and TIM3 expression in the mouse spleen; however, the PD-1/TIM3 levels were not correlated with resistance to CAR T cells, because CAR T cells eradicated AML in the xenograft and PDX models. T-cell suppression from Tregs was not observed, because of the robust elimination of the leukemia. Therefore, the results demonstrate that BissCAR T cells can effectively eradicate the double-positive PD AML cells in this clinically relevant model.

**Example 6: Combinatory BissCAR T Cells Targeting CD13 and TIMS have Reduced Toxicity to HSCs In Vivo**

The impact of BissCAR T cells on normal human HSCs was also investigated. Humanized immune system (HIS) mice were used to assess hematopoietic toxicity of BissCAR T cells (FIG. 7E). NSG mice were conditioned with busulfan and engrafted with bone marrow CD34<sup>+</sup> cells from a normal adult donor, followed by treatment with BissCAR, Nb157 CAR, or UTD T cells 4 weeks later. Bone marrow from these mice was collected for analysis 3 weeks after treatment. Nb157 CART cells almost completely depleted CD34<sup>+</sup>CD38<sup>-</sup> HSCs, CD34<sup>+</sup>CD38<sup>+</sup> myeloid progenitors, and peripheral monocytes (FIGS. 7F-7I). Notably, BissCAR T cells significantly reduced the toxicity to HSCs, retaining ~50% of the human HSC-enriched population and the myeloid progenitors of normal control mice (FIGS. 7F-7H). Moreover, BissCAR T cells significantly reduced the monocytes in peripheral blood and allowed the protection of part of the monocytes in peripheral blood in BissCAR T-cell-injected mice compared with Nb157 CAR T-cell-injected mice (FIG. 7I).

Together, these results indicate that BissCAR T cells effectively eradicate RD AML cells (FIGS. 7B-7D) and have much reduced toxicity to sensitive human HSCs (FIGS. 7F-7I), suggesting BissCAR T cells as a valuable approach to treat human AML with reduced and tolerable hematopoietic toxicity.

**Example 7: Generation of Unique Individual Anti-TIM3 VHHS**

Multiple novel VHHS targeting human TIM-3 were generated herein. Briefly, 3 llamas were immunized with purified human TIM-3 protein 4 times (Caprolitics, Hardwick, MA). Peripheral blood mononuclear cells (PBMCs) were isolated, RNA extracted and cDNA synthesis performed as previously described (He et al., *Blood*. 2020; 135 (10): 713-723). Nanobody encoding fragments were amplified from llama PBMC cDNA with PCR primers, and then cloned into phage display vector pCOMB3X as previously described (He et al., *Blood*. 2020; 135 (10): 713-723). The resulting phage library was generated by infection with VCSM13 helper phage and PEG-precipitated from the bacterial supernatant in preparation for cell-surface selection, followed by panning on control NB4 cells and NB4 cells-expressing human TIM-3, using procedures as previously described (He et al., *Blood*. 2020; 135 (10): 713-723). Among multiple clones selected, three phage clones, i.e. VHH30, 32, and 33 showed specific binding to NB4-expressing human TIM-3, but not the TIM-3 negative NB4 cells (FIG. 9).

**Example 8: Sequences of the Isolated VHHS Targeting Human TIM-3 Protein**

To generate VHHS targeting human TIM-3, human recombinant extracellular TIM-3 purified from HEK293 cells was used to immunize llamas, and the resulting VHH phage display libraries were constructed. The phage VHH clones targeting TIM-3 were screened by panning through TIM-3 expressing NB4 cells. The positive clones specifically binding to TIM-3 expressing NB4 cells were isolated. DNA encoding each of the VHHS, including VHH30, 32, and 33, were sequenced. The sequences were consistent with the predicted sequences for VHHS. Each of the VHHS, with both nucleotide and amino acid sequences, are listed below.

**Sequences of Isolated VHHS Targeting Human TIM-3 Protein:**

**Anti-TIM3 VHH12 nucleotide sequence**  
(SEQ ID NO: 19):  
CAGGTGCAGCTGCAGGAGTCTGGAGGAGATTGGTGAGACTGGGACT  
CTCTGAGACTCTCTGTGTAGTCCTGGAGGCACCTTCAGAAACTATGTT  
TATGGGCTGGTCCGCCAGGCTCCAGGGAGGAGCGTGAGTTGTGCT  
GCTATGAACGGAGTGGCGGCATCACAGTCTATGCAGACTCCGTGAAGG  
GCCGATTCAACCATCTCCAGAGACAACGCCAAGAACGCGGTGTATCTGCA  
AATGGGCAGCCTGAAACCTGGCGACACGGCGTTTATTACTGTGCAGCT  
GCTGCAATCGATGGTGGAACCGTCAGAAGCATTAAACAGTTATGCCTACT  
GGGGCCAGGGGACCCAGGTACCGTCTCCTCAGCGGCCACTAGT

**Anti-TIM3 VHH12 amino acid sequence**  
(SEQ ID NO: 20):  
QVQLQESGGGLVQTGDSLRLSCVSGGTFRNYVMGWRQAPGKEREFSVS  
AMNWSGGITVYADSVKGRTISRDNAKNAVYLQMGSLKPGDTAVYYCAA  
AAIDGGTVRSINSYAYWQGTQVTVSSAAATS

US 12,383,581 B2

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Anti-TIM3 VHH13 nucleotide sequence  
(SEQ ID NO: 21):  
CAGGTGCAGCTCAGGAGTCTGGAGGAGATTGGTGCAGGCTGGGGCT  
CTCTGAGCCTCTCTGTGCAGCCCTCTGGACGCACCTCAAGAACTATCT  
CATGGCCTGGTTCGCCAGACTCCAGGGAGGAGCGTGAGTTGTGGCA  
GCTATTACTCAGCTGGTACTAGATCATTAAATGAAGACTTCGTGAAGG  
GCCGATTACCATCTCCAGGGACAACGCCAAGAACACGGTGTATCTGCA  
AATGAACGACCTGAAAATGACGACACGGCGTTATTCTGTGCAGCA  
AGCCTACAGAGTGGGGGGTCACTACGGTACCGAAGTATGACTATTGGG  
GCCAGGGACCCAGGTACCCTCCTCAGCGGCCACTAGT

Anti-TIM3 VHH13 amino acid sequence  
(SEQ ID NO: 22):  
QVQLQESGGGLVQAGGSLSLSCAASGRTFKNYLMAWFRQAPGKEREVVA  
AITQLGTRSLNEDFVKGRFTISRDNAKNTVYLQMNDLKTDITGVYSCAA  
SIQSGGSLRYAKYDYWGQGTQVTVSSAAATS

Anti-TIM3 VHH28 nucleotide sequence  
(SEQ ID NO: 23):  
CAGGTGCAGCTCAGGAGTCTGGGGAGGATTGGTGCAGGCTGGGGCT  
CTCTGAGACTCTCTGTGCAGCCCTCTGAAGGCACCGTCAGCACCTACAC  
CATGGCCTGGTTCGCCAGGCTCCAGGGAGGAGCGTGAGTTGTAGCC  
AGGATTACTGGTGTAGTACGGCTGTGAAGGGCCGGTCACCTTCTCCA  
GAGACGAGCCCCAAAACACAGTGTATCTGCAAATGAACAGCCTGAAACC  
TGAGGACACGGCGTCTATTACTGCGGGCACACTATTGGGTGGTCGT  
CCAGATATGCCACTAGTATCAATACTTGGCCAGGGACCCAGGTCA  
CCGTCTCCTCAGCGGCCACTAGT

Anti-TIM3 VHH28 amino acid sequence  
(SEQ ID NO: 24):  
QVQLQESGGGLVQAGGSLSLSCAASEGTVSTYTMWFRQAPGKEREVVA  
RITGVSTAVKGRFTFSRDEPKNTVYLQMNSLKPEDTAVYYCAAHYLGGR  
PDMPTQYQYLQOGTQVTVSSAAATS

Anti-TIM3 VHH30 nucleotide sequence  
(SEQ ID NO: 25):  
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CTCTGAGACTCTCTGTGCAGCCCTCTGGATTACGGTTGGTAGTTATGT  
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GATCTGACATACTATCGTACTGGTGGTAGGTTACAGATAACGCTAATG  
GATATGCGTACTGGGCCAGGGTACCCAGGTACCGTCTCCTCAGCGGC  
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Anti-TIM3 VHH30 amino acid sequence  
(SEQ ID NO: 26):  
QVQLQESGGGLVQAGGSLSLSCAASGFTFGSYVMGWFRQAPGKEREVVA  
SISTSGGITSYADSVKGRFTVSRDNAKNTVYLQMNSLKPEDTAVYYCAR  
DLTYYRTGGRLPDNANGYAYWGQGTQVTVSSAAATS

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Anti-TIM3 VHH32 nucleotide sequence  
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5 CTCTAAATCTCTCTGTGCAGCTCTGGAAAGTCCTCAGACTCTATAC  
CGTCGGCTGGCACCGCCAGGCCAGGGAAAGCAGCGCAGTTGGTCGCA  
TGGATTAGTGGTGCAGCACAAACTATCATTGTCGTGAAGGGCC  
10 GATTACCATCTCCAGAGACAACGCCAAGAACACGGCACTCTGCAAAT  
GAACAACCTGGCACCTGAAGACACGGCGTCTATTACTGTAATCTACTG  
AACTACTGGGCCAGGGACCCAGGTACCGTCTCCTCAGCGCCGCCA  
15 CTAGT

Anti-TIM3 VHH32 amino acid sequence  
(SEQ ID NO: 28):  
QVQLQESGGGLVQAGGSLSLSCAASGSSFRLYTVGWHRQAPGKQRELVA

20 WISGAGSTNYHSSVKGRFTISRDNAKNTALLQMNNLAPEDTAVYYCNLL  
NYWGQGTQVTVSSAAATS

Anti-TIM3 VHH33 nucleotide sequence  
(SEQ ID NO: 29):  
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CTCTGAGACTCTCTGTGCAGCTCTGGACTCACGCCGATGCTTATGT  
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30 TGTATTAGTCTAGTGGTGTACTACAGCTATCCAGACTCCGTGAAGG  
GCCGATTACCATCTCCAGAGACAATGCCAAGAACACGGTGTACCTGCA  
AATGAACAGCCTGAAACCTGAGGACACGGCGTTATTACTGTGCGGCA  
35 GTTGCAGGCCGCTGGTGTACTACGGCATGAACTACTACGGCAAAGGGA  
CCCAGGTACCGTCTCCAGCGGCCACTAGT

Anti-TIM3 VHH33 amino acid sequence  
(SEQ ID NO: 30):  
40 QVQLQESGGGLVQPGGSLRLSCAVSGLTPDAYVMGWFRQAPGKEREVVS  
CISPSGGTTSYPDSVKGRFTISRDNAKNTVYLQMNSLKPEDTGVYYCAA  
VAGRWCODYGMNYYGKGTQVTVSSAAATS

45 Anti-TIM3 VHH38 nucleotide sequence  
(SEQ ID NO: 31):  
CAGGTGCAGCTCAGGAGTCTGGGGAGGTTGGTGCAGGCTGGGACT  
CTCTGAGACTCTCTGTGCAGCTGGACGCACGTTCACTGCGTCAACCTT  
50 GGGCTGGTCCGCCAGTCTCCAGGGAGGAGCGTGAGTTGTGCGCAGCG  
ATTAGTTGGTGGCGTGGTAGGCACTATGGGACTCCGTGAAGGGCC  
GATTACCATCTCCAGAGACAACCCAAGAACGATCAATCTGCAAAT  
55 GAATAGCCTGAAACCTGAGGACACGGCGTTATTACTGTGACAGGCC  
CAATTGATGGCGCAGACGGCGAGATGACTATGACAACCTGGGTCAAGGCC  
GGACCCAGGTACCGTCTCCAGCGGCCACTAGT

60 Anti-TIM3 VHH38 amino acid sequence  
(SEQ ID NO: 32):  
QVQLQESGGGLVQAGDSLRLSCAVGRTFSASTLGWFRQSPGKEREVVA  
ISWWRGAEAYYGSVKGRTFISRDNTKTTINLQMNSLKPEDTAVYYCARA  
65 QFDGATRADDYDNWGQGTQVTVSSAAATS

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**Example 9: Generation of CD13/TIM-3 VHVs Bi-CARs Using Newly Generated Anti-TIM3 VHVs, and Evaluation of the biCARs on Primary Human T Cells**

To generate more choices and better CD13-TIM-3 biCARs, each of the three new VHVs targeting TIM-3 were cloned downstream of the 2A sequence, as shown in FIG. 10A. The resulting constructs were packaged into lentiviruses, which were transduced to human primary T cells. Flow cytometry analysis showed that transduction of T cells with each of the biCARs resulted in expression of the CARs on the T cell surface that bind both CD13 protein antigen (FIG. 10B) and TIM-3 (FIG. 10C). Sequences for each of the Bi-CARs, i.e. Bi-CAR2 VHH12 (SEQ ID NO: 33), VHH13 (SEQ ID NO: 34), VHH28 (SEQ ID NO: 35), VHH30 (SEQ ID NO: 36), VHH32 (SEQ ID NO: 37), VHH33 (SEQ ID NO: 38), and VHH38 (SEQ ID NO: 39) are provided herein. Together, these results demonstrated that each of the biCARs are expressed on the T cell surface, and are functionally capable of binding both targets, CD13 and TIM-3.

The bispecific CARs demonstrated in vitro killing against NB4 (FIG. 11A) and NB4-TIM3 (FIG. 11B) cells.

The various bispecific CARTs were also capable of suppressing tumors in vivo (FIGS. 12A-12E). The anti-tumor effects of bispecific CAR T cells against NB4-CD13KO (FIG. 12A), NB4 (FIG. 12B) and NB4-TIM3 (FIG. 12C) tumors were evaluated. Various NB4 cells were injected subcutaneously into each flank of a NSG mice ( $n=3$ ), and the indicated CARTs ( $5 \times 10^6$ ) were injected into each mouse via tail vein at 7 days after tumor cell injection. The number of human T cells in peripheral blood of mice bearing NB4-CD13KO, NB4 and NB4-TIM3 tumors was measured by flow cytometry 7 days (FIG. 12D) and 14 days (FIG. 12E) after T cell infusion.

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The potential toxicity of the bispecific CAR T cells against human bone marrow CD34+ cells was evaluated (FIGS. 13A-13B). BM CD34+ cells (5000 per well) were co-cultured with the indicated T cells (0.3:1, 1:1, 3:1) for 4 hours. Cells were transferred into 12-well plates and cultured in MethoCult™ H4435 Enriched medium. Two weeks later, the number of clones was measured. FIG. 13A shows the number of colonies from control (UTD) or the bispecific CART treated plates. FIG. 13B is a linear graph comparing the dose-dependent effect of the CARTs based on colony number. Overall, the results demonstrated that generation of multiple TIM-3 VHVs allowed construction of CD13-TIM3 biCARs that express the split CAR components to specifically target CD13-TIM-3 dual positive cancer cells, yet reduce the toxicity to normal cells with single target.

#### Other Embodiments

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

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<211> LENGTH: 18  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: VHH157 CDR2

<400> SEQUENCE: 4

Gly	Ile	Tyr	Pro	Ser	Asp	Gly	Lys	Thr	Arg	Tyr	Ala	Asp	Phe	Val	Lys
1				5			10		15						

Gly Arg

<210> SEQ ID NO 5  
<211> LENGTH: 9  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: VHH157 CDR3

<400> SEQUENCE: 5

Ala	Arg	Gly	Ile	Thr	Gly	Leu	Gly	Pro
1				5				

<210> SEQ ID NO 6  
<211> LENGTH: 249  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 scFv

<400> SEQUENCE: 6

Gln	Val	Gln	Leu	Gln	Gln	Pro	Gly	Ala	Glu	Leu	Val	Lys	Pro	Gly	Ala
1				5			10		15						

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr

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20	25	30
Asn Met His Trp Ile Lys Gln Thr Pro Gly Gln Gly Leu Glu Trp Ile		
35	40	45
Gly Asp Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe		
50	55	60
Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr		
65	70	75
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys		
85	90	95
Ala Arg Val Gly Ala Phe Pro Met Asp Tyr Trp Gly Gln Gly Thr		
100	105	110
Ser Val Thr Val Ser Ser Gly Gly Ser Gly Gly Ser Gly Ser		
115	120	125
Gly Gly Gly Ser Gly Gly Ser Asp Ile Val Leu Thr Gln		
130	135	140
Ser Pro Ala Ser Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser		
145	150	155
Cys Arg Ala Ser Glu Ser Val Glu Tyr Tyr Gly Thr Ser Leu Met Gln		
165	170	175
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Ala		
180	185	190
Ala Ser Asn Val Glu Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly		
195	200	205
Ser Gly Thr Asp Phe Ser Leu Asn Ile His Pro Val Glu Glu Asp Asp		
210	215	220
Ile Ala Ile Tyr Phe Cys Gln Gln Ser Arg Lys Asp Pro Ser Thr Phe		
225	230	235
Gly Gly Gly Thr Lys Leu Glu Ile Lys		
245		

&lt;210&gt; SEQ ID NO 7

&lt;211&gt; LENGTH: 747

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: TIM3 scFv

&lt;400&gt; SEQUENCE: 7

caggtgcaac tgcagcagcc tggggcttag ctggtaagc ctggggcctc agtgaagatg	60
tcctgcaagg cttctggcta cacatttacc agttacaata tgcactggat aaaggcagaca	120
cctggacagg gccttggatg gattggagat atttatccag gaaatggtga tacttcctac	180
aatcagaaat tcaaaggcaa ggccacattt actgcagaca aatcctccag cacagtctac	240
atgcagctca gcagcctgac atctgaggac tctgcggctt attactgtgc aagagtgggg	300
ggtgcccttc ctagggacta ctggggtcaa ggaacctcgat tcaccgtctc ctcaggaggc	360
ggaggatctg gccccggagg aagtggcgga gggggatcgag ggggaggccg atctgacatt	420
gtgctcaccc aatctccagc ttctttggct gtgtctctag ggcagagagc caccatctcc	480
tgcagagcca gtgaaaagtgt tgaatattat ggcacaaggta taatgcagtg gtaccaacag	540
aaaccaggac agccacccaa actcctcatc tatgctgcat ccaacgtaga atctgggtc	600
cctgccaggt ttagtggcag tgggtctggg acagactca ggcctcaacat ccatactgtg	660
gaggaggatg atattgcaat atatttctgt cagcaaagta ggaaggatcc ttgcacgttc	720
ggtgaggcga ccaagctgga gatcaaa	747

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<210> SEQ ID NO 8  
<211> LENGTH: 10  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 CDR1

&lt;400&gt; SEQUENCE: 8

Gly Tyr Thr Phe Thr Ser Tyr Asn Met His  
1 5 10

<210> SEQ ID NO 9  
<211> LENGTH: 17  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 CDR2

&lt;400&gt; SEQUENCE: 9

Asp Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys  
1 5 10 15

Gly

<210> SEQ ID NO 10  
<211> LENGTH: 9  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 CDR3

&lt;400&gt; SEQUENCE: 10

Val Gly Gly Ala Phe Pro Met Asp Tyr  
1 5

<210> SEQ ID NO 11  
<211> LENGTH: 248  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: PNE scFv

&lt;400&gt; SEQUENCE: 11

His Ala Ala Arg Pro Asp Ala Val Val Thr Gln Glu Ser Ala Leu Thr  
1 5 10 15

Ser Ser Pro Gly Glu Thr Val Thr Leu Thr Cys Arg Ser Ser Thr Gly  
20 25 30

Ala Val Thr Thr Ser Asn Tyr Ala Ser Trp Val Gln Glu Lys Pro Asp  
35 40 45

His Leu Phe Thr Gly Leu Ile Gly Gly Thr Asn Asn Arg Ala Pro Gly  
50 55 60

Val Pro Ala Arg Phe Ser Gly Ser Leu Ile Gly Asp Lys Ala Ala Leu  
65 70 75 80

Thr Ile Thr Gly Ala Gln Thr Glu Asp Glu Ala Ile Tyr Phe Cys Val  
85 90 95

Leu Trp Tyr Ser Asp His Trp Val Phe Gly Gly Thr Lys Leu Thr  
100 105 110

Val Leu Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly  
115 120 125

Gly Ser Gly Gly Gly Ser Asp Val Gln Leu Gln Glu Ser Gly Pro  
130 135 140

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Gly	Leu	Val	Ala	Pro	Ser	Gln	Ser	Leu	Ser	Ile	Thr	Cys	Thr	Val	Ser
145						150				155				160	
Gly	Phe	Leu	Leu	Thr	Asp	Tyr	Gly	Val	Asn	Trp	Val	Arg	Gln	Ser	Pro
	165						170				175				
Gly	Lys	Gly	Leu	Glu	Trp	Leu	Gly	Val	Ile	Trp	Gly	Asp	Gly	Ile	Thr
	180						185				190				
Asp	Tyr	Asn	Ser	Ala	Leu	Lys	Ser	Arg	Leu	Ser	Val	Thr	Lys	Asp	Asn
	195					200				205					
Ser	Lys	Ser	Gln	Val	Phe	Leu	Lys	Met	Asn	Ser	Leu	Gln	Ser	Gly	Asp
	210					215				220					
Ser	Ala	Arg	Tyr	Tyr	Cys	Val	Thr	Gly	Leu	Phe	Asp	Tyr	Trp	Gly	Gln
	225					230				235			240		
Gly	Thr	Thr	Leu	Thr	Val	Ser	Ser								
						245									

<210> SEQ ID NO 12  
<211> LENGTH: 744  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: PNE scFv

&lt;400&gt; SEQUENCE: 12

catggcgcta	gacctgtatgc	cgtcgatgacc	caggaaagcg	ccctgacaag	cagccctggc	60
gagacagtga	ccctgacactg	cagatctago	acaggcgccg	tgaccaccag	caactacgcc	120
agctgggtgc	aggaaaagcc	cgaccacctg	ttcacccggc	tgatcggccg	caccaacaat	180
agagcacctg	gcgtgcccgc	cagattcago	ggetctctga	tcggagataa	ggccgcctg	240
accatcaactg	gccccagac	agaggacgag	gccatctact	tttgcgtgct	gtggtacagc	300
gaccactggg	tgttcggccg	aggcaccaag	ctgacagtgc	tggcggagg	cggaggatct	360
ggcgccggag	gaagtggccg	agggggatca	gggggaggcg	gatctgatgt	gcagctgcag	420
gaatctggcc	caggactggt	ggcccttagc	cagagcctga	gcatcacctg	taccgtgtcc	480
ggcttcctgc	tgaccgacta	cgcgctgaaac	tgggtgcgc	agtctctgg	caagggcctg	540
gaatggctgg	gagtgatctg	gggacacgga	atcacccact	acaactccgc	cctgaagtcc	600
cggctgagcg	tgaccaagga	caacagcaag	agecagggtg	tcctgaagat	gaacagctg	660
cagagcggcg	acageccccg	gtactattgt	gtgaccggcc	tgttcgacta	ctggggccag	720
ggcacaaccc	tgaccgtgtc	tagc				744

<210> SEQ ID NO 13  
<211> LENGTH: 477  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 CAR

&lt;400&gt; SEQUENCE: 13

Met	Ala	Leu	Pro	Val	Thr	Ala	Leu	Leu	Pro	Leu	Ala	Leu	Leu		
1						5			10			15			
His	Ala	Ala	Arg	Pro	Gly	Ser	Ala	Ala	Gln	Ala	Ala	Gln	Val	Gln	Leu
						20			25			30			
Gln	Gln	Pro	Gly	Ala	Glu	Leu	Val	Lys	Pro	Gly	Ala	Ser	Val	Lys	Met
						35			40			45			
Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Thr	Phe	Thr	Ser	Tyr	Asn	Met	His	Trp
						50			55			60			

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Ile Lys Gln Thr Pro Gly Gln Gly Leu Glu Trp Ile Gly Asp Ile Tyr  
65 70 75 80

Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala  
85 90 95

Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr Met Gln Leu Ser  
100 105 110

Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Val Gly  
115 120 125

Gly Ala Phe Pro Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val  
130 135 140

Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly  
145 150 155 160

Ser Gly Gly Gly Ser Asp Ile Val Leu Thr Gln Ser Pro Ala Ser  
165 170 175

Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Arg Ala Ser  
180 185 190

Glu Ser Val Glu Tyr Tyr Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln  
195 200 205

Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Ala Ala Ser Asn Val  
210 215 220

Glu Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp  
225 230 235 240

Phe Ser Leu Asn Ile His Pro Val Glu Glu Asp Asp Ile Ala Ile Tyr  
245 250 255

Phe Cys Gln Gln Ser Arg Lys Asp Pro Ser Thr Phe Gly Gly Thr  
260 265 270

Lys Leu Glu Ile Lys His Met Gly Gln Ala Gly Gln Ser Gly Glu Ser  
275 280 285

Lys Tyr Gly Pro Pro Cys Pro Pro Cys Pro Ala Ser Tyr Ile Trp Ala  
290 295 300

Pro Leu Ala Gly Thr Cys Gly Val Leu Leu Leu Ser Leu Val Ile Thr  
305 310 315 320

Leu Tyr Cys Lys Arg Gly Arg Lys Lys Leu Leu Tyr Ile Phe Lys Gln  
325 330 335

Pro Phe Met Arg Pro Val Gln Thr Thr Gln Glu Glu Asp Gly Cys Ser  
340 345 350

Cys Arg Phe Pro Glu Glu Glu Gly Gly Cys Glu Leu Arg Val Lys  
355 360 365

Phe Ser Arg Ser Ala Asp Ala Pro Ala Tyr Lys Gln Gly Gln Asn Gln  
370 375 380

Leu Tyr Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu  
385 390 395 400

Asp Lys Arg Arg Gly Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg  
405 410 415

Lys Asn Pro Gln Glu Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met  
420 425 430

Ala Glu Ala Tyr Ser Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly  
435 440 445

Lys Gly His Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp  
450 455 460

Thr Tyr Asp Ala Leu His Met Gln Ala Leu Pro Pro Arg  
465 470 475

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<210> SEQ ID NO 14  
<211> LENGTH: 1434  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 CAR

<400> SEQUENCE: 14

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atggccttac cagtgaccgc cttgtcctg ccgctggcct tgctgtccca cgccgccagg      60
ccgggatccg cggcccaggc ggcccaggtg caactgcagc agcctggggc tgagctggtg     120
aaggcctgggg ctcagtgaa gatgtcctgc aaggcttctg gctacacatt taccagttac    180
aatatgcact ggataaaagca gacacctgga cagggcctgg aatggattgg agatatttat   240
ccaggaaatg gtgatacttc ctacaatcag aaattcaaag gcaaggccac attgactgca   300
gacaaaatcct ccagcacagt ctacatgcag ctcagcagcc tgacatctga ggactctgcg  360
gtctattact gtgcaagagt ggggggtgcc tttcctatgg actactgggg tcaaggaacc  420
tcagtcacccg ttccttcagg aggccggagga tctggccggc gaggaagtgg cggagggggga 480
tcagggggag gcggatctga cattgtgctc acccaatctc cagtttctt ggctgtgtct  540
ctagggcaga gagccaccat ctcctgcaga gccagtgaaa gtgttgaata ttatggcaca  600
agtttaatgc agtggtacca acagaaaccca ggacagccac ccaaactcct catctatgct 660
gcatccaacg tagaatctgg ggtccctgcc aggttttagtgc cagtgccggc tgggacagac 720
ttcagcctca acatccatcc tgtggaggag gatgatattt caatatattt ctgtcagcaa 780
agtaggaagg atccttcgac gttegggtgga ggccaccaagc tggagatcaa acatatggc 840
caggccggcc agtccggaga gagcaagtac ggccctccct gcccccttg ccctgctagc 900
tacatctggg cgccttggc cgggacttgtt ggggtccctc tcctgtcaact ggttatcacc 960
ctttaactgca aacggggcag aaagaaactc ctgtatataat tcaaacaacc atttatgaga 1020
ccagtacaaa ctactcaaga ggaagatggc tgttagctgcc gatttccaga agaagaagaa 1080
ggaggatgtg aactgagagt gaagttcago aggagcgcag acgcccccggtt gtaaagcag 1140
ggccagaacc agctctataa cgactcaat ctaggacgaa gagaggagta cgatgtttt 1200
gacaagagac gtggccggga ccctgagatg gggggaaagc cgagaaggaa gaaccctcag 1260
gaaggcctgt acaaataact gcagaaagat aagatgggg aggccctacag tgagattgg 1320
atgaaaggcg agcgccggag gggcaagggg cacgatggcc tttaccaggg tctcagtaca 1380
gccaccaagg acacctacga cgccttcac atgcaggecc tgccccctcg ctaa 1434

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<210> SEQ ID NO 15  
<211> LENGTH: 733  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 CD13 biCAR

<400> SEQUENCE: 15

```

Met Ala Leu Pro Val Thr Ala Leu Leu Pro Leu Ala Leu Leu
1          5           10          15

His Ala Ala Arg Pro Gly Ser Ala Ala Gln Ala Ala Gln Val Gln Leu
20         25           30

Gln Glu Ser Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Ser Leu
35         40           45

Ser Cys Thr Ala Ser Gly Phe Thr Phe Ser Ser Tyr Ser Met Ala Trp
50         55           60

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Val Arg Gln Ala Pro Gly Lys Gly Pro Glu Trp Val Ser Gly Ile Tyr  
 65 70 75 80  
 Pro Ser Asp Gly Lys Thr Arg Tyr Ala Asp Phe Val Lys Gly Arg Phe  
 85 90 95  
 Ser Ile Ser Arg Asp Asn Ala Lys Asn Met Leu Tyr Leu Gln Met Asn  
 100 105 110  
 Asn Leu Glu Pro Glu Asp Thr Ala Leu Tyr Tyr Cys Ala Arg Gly Ile  
 115 120 125  
 Thr Gly Leu Gly Pro Arg Gly Gln Gly Thr Gln Val Thr Val Ser Ser  
 130 135 140  
 Ala Ala Ala Thr Ser Gly Gln Thr Val Ser Ser Glu Ser Lys Tyr Gly  
 145 150 155 160  
 Pro Pro Cys Pro Pro Cys Pro Tyr Ile Trp Ala Pro Leu Ala Gly Thr  
 165 170 175  
 Cys Gly Val Leu Leu Leu Ser Leu Val Ile Thr Leu Tyr Cys Arg Val  
 180 185 190  
 Lys Phe Ser Arg Ser Ala Asp Ala Pro Ala Tyr Lys Gln Gly Gln Asn  
 195 200 205  
 Gln Leu Tyr Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val  
 210 215 220  
 Leu Asp Lys Arg Arg Gly Arg Asp Pro Glu Met Gly Gly Lys Pro Arg  
 225 230 235 240  
 Arg Lys Asn Pro Gln Glu Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys  
 245 250 255  
 Met Ala Glu Ala Tyr Ser Glu Ile Gly Met Lys Gly Glu Arg Arg Arg  
 260 265 270  
 Gly Lys Gly His Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys  
 275 280 285  
 Asp Thr Tyr Asp Ala Leu His Met Gln Ala Leu Pro Pro Arg Leu Glu  
 290 295 300  
 Gly Ser Gly Ala Thr Asn Phe Ser Leu Leu Lys Gln Ala Gly Asp Val  
 305 310 315 320  
 Glu Glu Asn Pro Gly Pro Pro Arg Met Ala Leu Pro Val Thr Ala Leu  
 325 330 335  
 Leu Leu Pro Leu Ala Leu Leu His Ala Ala Arg Pro Gly Ser Ala  
 340 345 350  
 Ala Gln Ala Ala Gln Val Gln Leu Gln Gln Pro Gly Ala Glu Leu Val  
 355 360 365  
 Lys Pro Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr  
 370 375 380  
 Phe Thr Ser Tyr Asn Met His Trp Ile Lys Gln Thr Pro Gly Gln Gly  
 385 390 395 400  
 Leu Glu Trp Ile Gly Asp Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr  
 405 410 415  
 Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser  
 420 425 430  
 Ser Thr Val Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala  
 435 440 445  
 Val Tyr Tyr Cys Ala Arg Val Gly Gly Ala Phe Pro Met Asp Tyr Trp  
 450 455 460  
 Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly Ser Gly  
 465 470 475 480  
 Gly Gly Ser Gly Gly Ser Gly Gly Ser Gly Ser Asp Ile

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485	490	495
Val Leu Thr Gln Ser Pro Ala Ser	Leu Ala Val Ser	Gly Gln Arg
500	505	510
Ala Thr Ile Ser Cys Arg Ala Ser	Glu Ser Val Glu	Tyr Tyr Gly Thr
515	520	525
Ser Leu Met Gln Trp Tyr Gln Gln	Lys Pro Gly Gln	Pro Pro Lys Leu
530	535	540
Leu Ile Tyr Ala Ala Ser Asn Val	Glu Ser Gly Val	Pro Ala Arg Phe
545	550	555
Ser Gly Ser Gly Ser Gly Thr Asp	Phe Ser Leu Asn	Ile His Pro Val
565	570	575
Glu Glu Asp Asp Ile Ala Ile Tyr	Phe Cys Gln Gln	Ser Arg Lys Asp
580	585	590
Pro Ser Thr Phe Gly Gly Thr	Lys Leu Glu Ile	Lys His Met Gly
595	600	605
Lys His Leu Cys Pro Ser Pro	Leu Phe Pro Gly	Pro Ser Lys Pro Phe
610	615	620
Trp Val Leu Val Val Val	Gly Val Leu Ala	Cys Tyr Ser Leu Leu
625	630	635
Val Thr Val Ala Phe Ile Ile Phe	Trp Val Arg Ser	Lys Arg Ser Arg
645	650	655
Leu Leu His Ser Asp Tyr Met Asn	Met Thr Pro Arg	Arg Pro Gly Pro
660	665	670
Thr Arg Lys His Tyr Gln Pro	Tyr Ala Pro Pro Arg	Asp Phe Ala Ala
675	680	685
Tyr Arg Ser Lys Arg Gly Arg	Lys Lys Leu Leu	Tyr Ile Phe Lys Gln
690	695	700
Pro Phe Met Arg Pro Val Gln	Thr Thr Gln Glu	Asp Gly Cys Ser
705	710	715
Cys Arg Phe Pro Glu Glu Glu	Gly Cys Glu	Leu
725	730	

&lt;210&gt; SEQ ID NO 16

&lt;211&gt; LENGTH: 2202

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: TIM3 CD13 biCAR

&lt;400&gt; SEQUENCE: 16

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atggccttac cagtgaccgc cttgtcctg ccgctggct tgcgtctcca cgccgccagg   60
ccgggatccg cggcccaggc ggcccaggtg cagctgcagg agtctggggg aggcttggtg 120
cagcctgggg ggtctctgag cctctcctgt acagcctctg gattcacgtt cagtagttac 180
tccatggcct ggtccgcca ggctccaggg aagggacccc aatgggtctc agggatttac 240
ccttctgtat gtaagacaag gtatgcagac ttctgtgaagg gccgattcag catctccaga 300
gacaacgcca agaatatgtt gtatctgcaa atgaacaacc tgaaacctga ggacacggcc 360
ctatattact gtgcgagagg tatcacccga ttgggacccc gggccaggc gaccaggtc 420
accgtctcct cagcgccgc cactagtggc cagaccgtgt ctgcgagtc taagtacggc 480
cctccctgccc tccttgccc atacatctgg gcgccttgg cggggacttg tgggtcctt 540
ctcctgtcac tggttatcac ccttactgc agagtgaagt tcagcaggag cgcagacgcc 600
cccgcgatac agcaggccca gaaccagctc tataacgagc tcaatctagg acgaagagag 660

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gagtaacatg tttggacaa gagacgtggc cgggaccctg agatgggggg aaagccgaga	720
aggaagaacc ctcaggaagg cctgtacaat gaactgcaga aagataagat ggccggaggcc	780
tacagtgaga ttggatgaa aggccggcgc cggaggggca aggggcacga tggcattac	840
cagggtctca gtacagccac caaggacacc tacgacgcc ttcacatgca ggcctgccc	900
cctcgccctcg agggaaaggcg agtactaac ttcagccctgc tgaagcggc tggagacgtg	960
gaggagaacc ctggacctcc taggatggcc ttaccatgtc ccgccttgct cctgcgcgt	1020
gccttgcgtgc tccacgccc caggccggga tccgcggccc aggcggccca ggtcaactg	1080
cagcagectg gggctgagct ggtgaaggct ggggcctcg tgaagatgtc ctgcaaggct	1140
tctggctaca catttaccag ttacaatatg cactggataa agcagacacc tggacaggc	1200
ctggaatgga ttggagatat ttatccagga aatgggtataa cttcctacaa tcagaaattc	1260
aaaggcaagg ccacattgac tgccagacaaa tcctccagca cagtctacat gcagctcagc	1320
agcctgacat ctgaggactc tgccgtctat tactgtgcaaa gagtgggggg tgccttcct	1380
atggactact ggggtcaagg aacctcagtc accgtctcct caggaggccgg aggtctggc	1440
ggccggaggaa gtggccggagg gggatcaggg ggaggccggat ctgacattgt gctcacccaa	1500
tctccagctt ctttggctgt gtctctaggg cagagagcca ccattctcctg cagagccagt	1560
gaaaatgttg aatattatgg cacaagttt atgcagtggt accaacagaa accaggacag	1620
ccacccaaac tcctcatcta tgctgcattcc aacgttagaat ctggggtccc tgccaggatt	1680
atggcgcagtg ggtctggac agacttcagc ctcaacatcc atcctgtgga ggaggatgat	1740
attgcaatat atttctgtca gcaaagttagg aaggatcctt cgacgttcgg tggaggcacc	1800
aagctggaga tcaaacatata gggaaacac ctttgtccaa gtccccatt tccccgacct	1860
tctaaggccct tttgggtgct ggtgggtggtt ggtggagtcc tggcttgctta tagctgtca	1920
gttaacagtgg cctttattat tttctgggtg aggagtaaga ggagcaggct cctgcacagt	1980
gactacatga acatgactcc cccgcgcacc gggccacc ccaagcatcc ccagccctat	2040
gccccaccac gcgacttcgc agcctatcgc tccaaacggg gcagaaagaa actcctgtat	2100
atattcaaac aaccatttat gagaccagta caaaactactc aagagaaaga tggctgttagc	2160
tgcgcatttc cagaagaaga agaaggagga tgtgaactgt aa	2202

&lt;210&gt; SEQ ID NO 17

&lt;211&gt; LENGTH: 842

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: TIM3 PNE inducible bispecific CAR

&lt;400&gt; SEQUENCE: 17

Met Ala Leu Pro Val Thr Ala Leu Leu Pro Leu Ala Leu Leu			
1	5	10	15

His Ala Ala Arg Pro Gly Ser Ala Ala Gln Ala Ala Gln Val Gln Leu			
20	25	30	

Gln Gln Pro Gly Ala Glu Leu Val Lys Pro Gly Ala Ser Val Lys Met			
35	40	45	

Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn Met His Trp			
50	55	60	

Ile Lys Gln Thr Pro Gly Gln Gly Leu Glu Trp Ile Gly Asp Ile Tyr			
65	70	75	80

Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys Gly Lys Ala			
85	90	95	

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Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Val Tyr Met Gln Leu Ser  
 100 105 110  
 Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala Arg Val Gly  
 115 120 125  
 Gly Ala Phe Pro Met Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val  
 130 135 140  
 Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly  
 145 150 155 160  
 Ser Gly Gly Gly Ser Asp Ile Val Leu Thr Gln Ser Pro Ala Ser  
 165 170 175  
 Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Arg Ala Ser  
 180 185 190  
 Glu Ser Val Glu Tyr Tyr Gly Thr Ser Leu Met Gln Trp Tyr Gln Gln  
 195 200 205  
 Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Tyr Ala Ala Ser Asn Val  
 210 215 220  
 Glu Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Thr Asp  
 225 230 235 240  
 Phe Ser Leu Asn Ile His Pro Val Glu Glu Asp Asp Ile Ala Ile Tyr  
 245 250 255  
 Phe Cys Gln Gln Ser Arg Lys Asp Pro Ser Thr Phe Gly Gly Thr  
 260 265 270  
 Lys Leu Glu Ile Lys His Met Gly Lys His Leu Cys Pro Ser Pro Leu  
 275 280 285  
 Phe Pro Gly Pro Ser Lys Pro Phe Trp Val Leu Val Val Val Gly Gly  
 290 295 300  
 Val Leu Ala Cys Tyr Ser Leu Leu Val Thr Val Ala Phe Ile Ile Phe  
 305 310 315 320  
 Trp Val Arg Ser Lys Arg Ser Arg Leu Leu His Ser Asp Tyr Met Asn  
 325 330 335  
 Met Thr Pro Arg Arg Pro Gly Pro Thr Arg Lys His Tyr Gln Pro Tyr  
 340 345 350  
 Ala Pro Pro Arg Asp Phe Ala Ala Tyr Arg Ser Lys Arg Gly Arg Lys  
 355 360 365  
 Lys Leu Leu Tyr Ile Phe Lys Gln Pro Phe Met Arg Pro Val Gln Thr  
 370 375 380  
 Thr Gln Glu Glu Asp Gly Cys Ser Cys Arg Phe Pro Glu Glu Glu  
 385 390 395 400  
 Gly Gly Cys Glu Leu Leu Glu Gly Ser Gly Ala Thr Asn Phe Ser Leu  
 405 410 415  
 Leu Lys Gln Ala Gly Asp Val Glu Glu Asn Pro Gly Pro Pro Arg Met  
 420 425 430  
 Ala Leu Pro Val Thr Ala Leu Leu Pro Leu Ala Leu Leu His  
 435 440 445  
 Ala Ala Arg Pro Asp Ala Val Val Thr Gln Glu Ser Ala Leu Thr Ser  
 450 455 460  
 Ser Pro Gly Glu Thr Val Thr Leu Thr Cys Arg Ser Ser Thr Gly Ala  
 465 470 475 480  
 Val Thr Thr Ser Asn Tyr Ala Ser Trp Val Gln Glu Lys Pro Asp His  
 485 490 495  
 Leu Phe Thr Gly Leu Ile Gly Gly Thr Asn Asn Arg Ala Pro Gly Val  
 500 505 510

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Pro Ala Arg Phe Ser Gly Ser Leu Ile Gly Asp Lys Ala Ala Leu Thr  
515 520 525

Ile Thr Gly Ala Gln Thr Glu Asp Glu Ala Ile Tyr Phe Cys Val Leu  
530 535 540

Trp Tyr Ser Asp His Trp Val Phe Gly Gly Thr Lys Leu Thr Val  
545 550 555 560

Leu Gly Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly  
565 570 575

Ser Gly Gly Ser Asp Val Gln Leu Gln Glu Ser Gly Pro Gly  
580 585 590

Leu Val Ala Pro Ser Gln Ser Leu Ser Ile Thr Cys Thr Val Ser Gly  
595 600 605

Phe Leu Leu Thr Asp Tyr Gly Val Asn Trp Val Arg Gln Ser Pro Gly  
610 615 620

Lys Gly Leu Glu Trp Leu Gly Val Ile Trp Gly Asp Gly Ile Thr Asp  
625 630 635 640

Tyr Asn Ser Ala Leu Lys Ser Arg Leu Ser Val Thr Lys Asp Asn Ser  
645 650 655

Lys Ser Gln Val Phe Leu Lys Met Asn Ser Leu Gln Ser Gly Asp Ser  
660 665 670

Ala Arg Tyr Tyr Cys Val Thr Gly Leu Phe Asp Tyr Trp Gly Gln Gly  
675 680 685

Thr Thr Leu Thr Val Ser Ser Glu Ser Lys Tyr Gly Pro Pro Cys Pro  
690 695 700

Pro Cys Pro Tyr Ile Trp Ala Pro Leu Ala Gly Thr Cys Gly Val Leu  
705 710 715 720

Leu Leu Ser Leu Val Ile Thr Leu Tyr Cys Arg Val Lys Phe Ser Arg  
725 730 735

Ser Ala Asp Ala Pro Ala Tyr Lys Gln Gly Gln Asn Gln Leu Tyr Asn  
740 745 750

Glu Leu Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg  
755 760 765

Arg Gly Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro  
770 775 780

Gln Glu Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala  
785 790 795 800

Tyr Ser Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His  
805 810 815

Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp  
820 825 830

Ala Leu His Met Gln Ala Leu Pro Pro Arg  
835 840

<210> SEQ ID NO 18  
<211> LENGTH: 2529  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: TIM3 PNE inducible bispecific CAR

&lt;400&gt; SEQUENCE: 18

atggccttac cagtgaccgc cttgtccctg ccgcgtggct tgctgctcca cgccgccagg	60
ccgggatccg cggcccaggc ggccccagtg caactgcagc agcctggggc tgagctggtg	120
aaggctgggg cctcagtgaa gatgtcctgc aaggcttctg gctacacatt taccagttac	180

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aatatgact ggataaagca gacaccttgg cagggcctgg aatggattgg agatatttat  
ccaggaaatg gtgatacttc ctacaatcg aaattcaaaag gcaaggccac attgactgca  
gacaatccct ccagcacagt ctacatcgag ctcagcagcc tgacatctga ggactctgcg  
gtctattact gtgcaagagt ggggggtgcc tttctatgg actactgggg tcaaggaacc  
tcagtcaccc ttcctcagg aggccggagga tctggccggcg gaggaagtgg cgagggggga  
tcagggggag gcggatctga catttgctc acccaatctc cagcttctt ggctgtgtct  
ctagggcaga gagccaccat ctcctgcaga gccagtaaa gtgttaata ttatggcaca  
agtttaatgc agtggtagcca acagaaacca ggacagccac ccaaactctt catctatgt  
gcatccaacg tagaatctgg ggtccctgcc aggttagtg gcagtggtc tggacagac  
ttcagcctca acatccatcc tggggaggag gatgatattt caaatatattt ctgtcagcaa  
agtaggaagg atccttcgac gttcggtgg ggcaccaagc tggagatcaa acatatgggg  
aaacacccctt gtccaaatcc cctatcccc ggaccccttca agccctttt ggtgtgggt  
gtgggtgggt gagtccctggc ttgtatagc ttgttagaa cagtggcctt tattatccc  
tgggtgagga gtaagaggag caggctccctg cacagtact acatgaacat gactccccgc  
cgccccgggc ccacccggcaaa gcattaccag ccctatgccc caccacgcga cttcgagcc  
tatcgctcca aacggggcag aaagaaactc ctgtatata tcaaacaacc atttatgaga  
ccagtacaaa ctactcaaga ggaagatggc tggtagctgcc gattccaga agaagaagaa  
ggaggatgtg aactgtcga gggagccggc gctactaact tggccctgtc gaagcaggct  
ggagacgtgg aggagaaccc tggacccctt aggatggcgc tggctgtac agctctgt  
ctgcctctgg ccctgtgtc gcattccgcct agacctgtatcc cggctgtac ccagggaaagc  
gccctgacaa gcagccctgg cgagacagtg accctgaccc tggatcttag cacaggcgcc  
gtgaccacca gcaactacgc cagctgggtg cggaaaaagc cggaccaccc gttcacccggc  
ctgatcgccg gcaccaacaa tagacccat ggegtggcccg ccagattcag cggctctctg  
atcgagata aggccgcctt gaccatact ggcccccaga cagggacga ggcctatctac  
tttgcgtgc tgggtacag cgaccactgg gtgttccggc gaggccacaa gctgacagt  
ctggccggag gggggggatc tggccggggc ggaagttggc gggggggatc agggggggc  
ggatctgtatcc tggtagctgc ggaatctggc ccaggactgg tggcccttag ccagggcctg  
agcatcacct gtaccgtgtc cggctccctg ctgaccgact acggccgtaa ctgggtgcgc  
cagtctctg gcaaggccctt ggaatggctg ggagtgtatct gggccaccc aatcaccgac  
tacaactccg ccctgaagtc cgggctgagc gtgaccaagg acaacagccaa gagccagggt  
ttcctgaaga tgaacacgcct gcagagccggc gacagccccc ggtactattt tggtagccggc  
ctgttgcact actggggccca gggcacaacc ctgaccgtgt ctggcgtac taaatcgcc  
cctccctgcc ctccttgccc atacatctgg ggcgccttgg cggggacttg tggggccctt  
cteetgtcac tggttatcac ccttactgc agagtgaagt tggccggagg cgcagacgc  
ccggcgtaca agcaggccca gaaccagctc tataacgagc taaatctagg acgaagag  
gagttacgtatcc ttttggacaa gagacgtggc cgggaccctg agatgggggg aaagccgaga  
aggaagaacc ctcagggcagg cctgtacaat gaaatgcaga aagataagat ggcggaggcc  
tacagtgaga ttgggtatggaa aggcgcggc cggggggggc aggggcacga tggccctttac  
cagggtctca gtacagccac caaggacacc tacgacccccc ttcacatgca ggcctgccc  
cctcgctaa

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<210> SEQ ID NO 19  
<211> LENGTH: 390  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH12

<400> SEQUENCE: 19

```
caggtgcagc tgcaggagtc tggaggagga ttgggtcaga ctggggactc tctgagactc      60
tcctgttag tctctggagg cacttcaga aactatgtta tgggtcggtt ccgcaggct      120
ccagggaaagg agcgtgagtt tgggtctgtat atgaactgaa gtggcgcat cacagtctat      180
gcagactccg tgaagggccg attcaccatc tccagagaca acgccaagaa cgccgttat      240
ctgcaaatgg gcagcctgaa acctggcgac acggccgtt attactgtgc agctgctgca      300
atcgatggtg gaaccgtcag aagcattaac agttatgcct actggggcca ggggaccag      360
gtcaccgtct ctcagcgcc cgccactagt                                390
```

<210> SEQ ID NO 20  
<211> LENGTH: 130  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH12

<400> SEQUENCE: 20

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Leu	Val	Gln	Thr	Gly	Asp
1							5		10				15		

Ser	Leu	Arg	Leu	Ser	Cys	Val	Val	Ser	Gly	Gly	Thr	Phe	Arg	Asn	Tyr
						20		25			30				

Val	Met	Gly	Trp	Phe	Arg	Gln	Ala	Pro	Gly	Lys	Glu	Arg	Glu	Phe	Val
	35					40				45					

Ser	Ala	Met	Asn	Trp	Ser	Gly	Gly	Ile	Thr	Val	Tyr	Ala	Asp	Ser	Val
	50				55			60							

Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Ala	Val	Tyr
65					70			75			80				

Leu	Gln	Met	Gly	Ser	Leu	Lys	Pro	Gly	Asp	Thr	Ala	Val	Tyr	Tyr	Cys
					85			90		95					

Ala	Ala	Ala	Ile	Asp	Gly	Gly	Thr	Val	Arg	Ser	Ile	Asn	Ser	Tyr	
				100				105		110					

Ala	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser	Ala	Ala	
		115				120			125						

Thr	Ser
	130

<210> SEQ ID NO 21  
<211> LENGTH: 387  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH13

<400> SEQUENCE: 21

```
caggtgcagc tgcaggagtc tggaggagga ttgggtcagg ctggggctc tctgagcctc      60
tcctgtcagc ctcctggacg cacttcaag aactatcta tggctcggtt ccgcaggact      120
ccagggaaagg agcgtgagtt tgggtcgactt attactcagc ttggtaactag atcattaaat      180
gaagacttcg tgaagggccg attcaccatc tccagggaca acgccaagaa cacggtgtat      240
```

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ctgcaaatga acgacctgaa aactgacgac acggcggtt attcttgtc agcaagccta	300
cagagtgggg ggtcaactacg gtacgcgaag tatgactatt ggggccaggg gaccaggc	360
accgtctct cagcggccgc cactagt	387

<210> SEQ ID NO 22  
<211> LENGTH: 129  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH13

&lt;400&gt; SEQUENCE: 22

Gln Val Gln Leu Glu Ser Gly Gly Leu Val Gln Ala Gly Gly	
1 5 10 15	

Ser Leu Ser Leu Ser Cys Ala Ala Ser Gly Arg Thr Phe Lys Asn Tyr	
20 25 30	

Leu Met Ala Trp Phe Arg Gln Thr Pro Gly Lys Glu Arg Glu Phe Val	
35 40 45	

Ala Ala Ile Thr Gln Leu Gly Thr Arg Ser Leu Asn Glu Asp Phe Val	
50 55 60	

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr	
65 70 75 80	

Leu Gln Met Asn Asp Leu Lys Thr Asp Asp Thr Gly Val Tyr Ser Cys	
85 90 95	

Ala Ala Ser Leu Gln Ser Gly Gly Ser Leu Arg Tyr Ala Lys Tyr Asp	
100 105 110	

Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser Ala Ala Ala Thr	
115 120 125	

Ser

<210> SEQ ID NO 23  
<211> LENGTH: 369  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH28

&lt;400&gt; SEQUENCE: 23

caggtgcagc tgcaggagtc tgggggagga ttgggtcagg ctgggggctc tctgagactc	60
---	----

tcctgtcagc cctctgaagg cacccgtcagg acctacacca tggcctgggtt ccgccaggct	120
---	-----

ccagggaaagg agcgtgagtt tgttagccagg attactgggt ttagtacggc tgtgaaggc	180
--	-----

cggttcacct tctccagaga cgagccaaa aacacagtgt atctgaaat gaacagctg	240
--	-----

aaacctgagg acacggccgt ctattactgc gcccacact atttgggtgg tcgtccagat	300
--	-----

atgccgactc agtatcaata cttggggccag gggacccagg tcaccgtctc ctcagcggcc	360
--	-----

gccactagt	369
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<210> SEQ ID NO 24  
<211> LENGTH: 123  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH28

&lt;400&gt; SEQUENCE: 24

Gln Val Gln Leu Gln Glu Ser Gly Gly Leu Val Gln Ala Gly Gly	
1 5 10 15	

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Glu Gly Thr Val Ser Thr Tyr  
 20 25 30

Thr Met Ala Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Phe Val  
 35 40 45

Ala Arg Ile Thr Gly Val Ser Thr Ala Val Lys Gly Arg Phe Thr Phe  
 50 55 60

Ser Arg Asp Glu Pro Lys Asn Thr Val Tyr Leu Gln Met Asn Ser Leu  
 65 70 75 80

Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala Ala His Tyr Leu Gly  
 85 90 95

Gly Arg Pro Asp Met Pro Thr Gln Tyr Gln Tyr Leu Gly Gln Gly Thr  
 100 105 110

Gln Val Thr Val Ser Ser Ala Ala Ala Thr Ser  
 115 120

<210> SEQ ID NO 25  
<211> LENGTH: 402  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH30

<400> SEQUENCE: 25

caggtgcagc tgcaggagtc tgggggagga ttgggtgcagg ctgggggctc tctgagactc 60  
 tcctgtgcag cctctggatt cacgttttgtt agttatgtta tgggctggtt ccgccaggtt 120  
 ccagggaaagg agcgtgaatt tggcaagt attagtgacg gtgggtggcat aacatcttat 180  
 gcagactccg tgaagggcccg attcaactgtc tccagagaca acgccaagaa tacggtctac 240  
 ttacaaaatgaa acagcctgaa acctgaggac acggccgtttt attactgcgc acgagatctg 300  
 acataactatc gtactggtgg taggttacca gataacgcta atggatatgc gtactggggc 360  
 cagggtaccc aggtcacccgt ctcctcagcg gccgccacta gt 402

<210> SEQ ID NO 26  
<211> LENGTH: 134  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH30

<400> SEQUENCE: 26

Gln Val Gln Leu Gln Glu Ser Gly Gly Leu Val Gln Ala Gly Gly  
 1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Gly Ser Tyr  
 20 25 30

Val Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Phe Val  
 35 40 45

Ala Ser Ile Ser Thr Ser Gly Gly Ile Thr Ser Tyr Ala Asp Ser Val  
 50 55 60

Lys Gly Arg Phe Thr Val Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr  
 65 70 75 80

Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys  
 85 90 95

Ala Arg Asp Leu Thr Tyr Tyr Arg Thr Gly Gly Arg Leu Pro Asp Asn  
 100 105 110

Ala Asn Gly Tyr Ala Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser  
 115 120 125

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Ser Ala Ala Ala Thr Ser  
130

<210> SEQ ID NO 27  
<211> LENGTH: 348  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH32

&lt;400&gt; SEQUENCE: 27

cagggtgcagc	tgcaggagtc	tggaggaggo	ttgggtgcagg	ctggggggtc	tctaaatctc	60
tcctgtgcag	cctctggaag	ttccttcaga	ctctataccg	tcggctggca	ccgccaggcg	120
ccagggaaagc	agcgcgagtt	ggtcgcattgg	attagtggtg	ccccgcagcac	aaactatcat	180
tcgtccgtga	agggccgatt	caccatctcc	agagacaacg	ccaagaacac	ggcactctg	240
caaataaca	acctggcacc	tgaagacacg	gccgtctatt	actgtaatct	actgaactac	300
tggggccagg	ggacccaggt	caccgtctcc	tcagccggccg	ccactagt		348

&lt;210&gt; SEQ ID NO 28

<211> LENGTH: 116  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH32

&lt;400&gt; SEQUENCE: 28

Gln	Val	Gln	Leu	Gln	Glu	Ser	Gly	Gly	Gly	Ley	Val	Gln	Ala	Gly	Gly
1				5			10				15				

Ser	Leu	Asn	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Ser	Ser	Phe	Arg	Leu	Tyr
	20				25						30				

Thr	Val	Gly	Trp	His	Arg	Gln	Ala	Pro	Gly	Lys	Gln	Arg	Glu	Ley	Val
	35			40						45					

Ala	Trp	Ile	Ser	Gly	Ala	Gly	Ser	Thr	Asn	Tyr	His	Ser	Ser	Val	Lys
50			55						60						

Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ala	Lys	Asn	Thr	Ala	Ley	Leu
65				70				75			80				

Gln	Met	Asn	Asn	Leu	Ala	Pro	Glu	Asp	Thr	Ala	Val	Tyr	Tyr	Cys	Asn
	85					90			95						

Leu	Leu	Asn	Tyr	Trp	Gly	Gln	Gly	Thr	Gln	Val	Thr	Val	Ser	Ser	Ala
	100				105					110					

Ala	Ala	Thr	Ser
	115		

<210> SEQ ID NO 29  
<211> LENGTH: 378  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH33

&lt;400&gt; SEQUENCE: 29

cagggtgcagc	tgcaggagtc	tggggaggo	ttgggtgcagc	ctggggggtc	tctgagactc	60
tcctgtgcag	tctctggact	cacgcccgt	gcttatgtca	tgggtgggtt	ccgccaggcc	120
ccagggaaagg	agcgcgaggg	ggtctcatgt	attagtctca	gtgggtgtac	tacaagctat	180
ccagactccg	tgaaggggccg	attcaccatc	tccagagaca	atgccaagaa	cacgggtac	240
ctgcaaata	acagcctgaa	acctgaggac	acgggcgttt	attactgtgc	ggcagttgcg	300

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ggccgctgggt gtgactacgg catgaactac tacggcaaag ggaccaggc caccgtctcc      360
tcagcgcccg ccactagt                                         378
```

<210> SEQ ID NO 30  
<211> LENGTH: 126  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH33

<400> SEQUENCE: 30

```
Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1           5           10          15

Ser Leu Arg Leu Ser Cys Ala Val Ser Gly Leu Thr Pro Asp Ala Tyr
20          25          30

Val Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val
35          40          45

Ser Cys Ile Ser Pro Ser Gly Gly Thr Thr Ser Tyr Pro Asp Ser Val
50          55          60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Val Tyr
65          70          75          80

Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Gly Val Tyr Tyr Cys
85          90          95

Ala Ala Val Ala Gly Arg Trp Cys Asp Tyr Gly Met Asn Tyr Tyr Gly
100         105         110

Lys Gly Thr Gln Val Thr Val Ser Ser Ala Ala Ala Thr Ser
115         120         125
```

<210> SEQ ID NO 31  
<211> LENGTH: 381  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH38

<400> SEQUENCE: 31

```
caggtgcagc tgcaggagtc tgggggaggt ttgggtgcagg ctggggactc tctgagactc      60
tcctgtgcag tcggacgcac gttcagtgcg tcaaccttgg gctgggtccg ccagtctcca     120
ggaaaggagc gtgagtttgt cgcaagcgatt agttggtgcc gtggtgaggc atactatggg     180
gactccgtga agggccgatt caccatctcc agagacaaca ccaagacaac gatcaatctg    240
caaataatgataa gctgaaaacc tgaggacacg gccgtttatt actgtgcacg agcccaattt   300
gatggcgcga cacggccaga tgactatgac aactggggtc aggggaccac ggtcaccgtc    360
tcctcagcgcc cgccactag t                                         381
```

<210> SEQ ID NO 32  
<211> LENGTH: 127  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Anti TIM3 VHH38

<400> SEQUENCE: 32

```
Gln Val Gln Leu Gln Glu Ser Gly Gly Gly Leu Val Gln Ala Gly Asp
1           5           10          15

Ser Leu Arg Leu Ser Cys Ala Val Gly Arg Thr Phe Ser Ala Ser Thr
20          25          30
```

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Leu Gly Trp Phe Arg Gln Ser Pro Gly Lys Glu Arg Glu Phe Val Ala  
35 40 45

Ala Ile Ser Trp Trp Arg Gly Glu Ala Tyr Tyr Gly Asp Ser Val Lys  
50 55 60

Gly Arg Phe Thr Ile Ser Arg Asp Asn Thr Lys Thr Thr Ile Asn Leu  
65 70 75 80

Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Val Tyr Tyr Cys Ala  
85 90 95

Arg Ala Gln Phe Asp Gly Ala Thr Arg Ala Asp Asp Tyr Asp Asn Trp  
100 105 110

Gly Gln Gly Thr Gln Val Thr Val Ser Ser Ala Ala Ala Thr Ser  
115 120 125

&lt;210&gt; SEQ ID NO 33

&lt;211&gt; LENGTH: 9439

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: BiCAR VH12

&lt;400&gt; SEQUENCE: 33

```

gtgcacgagt gggttacate gaactggatc tcaacagcgg taagatccctt gagagtttc      60
gccccgaaga acgttttcca atgatgagca ctttaaagt tctgctatgt ggcgcggtat      120
tatcccgtat tgacgcgggg caagagcaac tcggtcgccc catacactat tctcagaatg      180
acttgggtga gtactcacca gtcacagaaa agcatcttac ggatggcatg acagtaagag      240
aattatgcag tgctgccata accatgagtg ataacactgc ggccaactt cttctgacaa      300
cgatcggagg accgaaggag ctaaccgctt tttgcacaa catggggat catgtactc      360
gccttgcatcg ttggaaaccg gagctgaatg aagccatacc aaacgcgag cgtgacacca      420
cgatgcctgt agcaatggca acaacgttgc gcaaactatt aactggcgaa ctacttactc      480
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**179****180**

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What is claimed is:

1. A bispecific chimeric antigen receptor (CAR) comprising a first antigen binding domain capable of binding CD13, a first intracellular domain, a second antigen binding domain capable of binding TIM-3, a transmembrane domain, and a second intracellular domain, wherein the first antigen binding domain comprises the amino acid sequence set forth in SEQ ID NO: 1.
2. The bispecific CAR of claim 1, wherein the first and/or second antigen binding domain is selected from the group consisting of an antibody, a nanobody, a Fab, and an scFv.
3. The bispecific CAR of claim 1, wherein the second antigen binding domain comprises the amino acid sequence set forth in SEQ ID NO: 6.
4. The bispecific CAR of claim 1, wherein the second antigen binding domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 20, 22, 24, 26, 28, 30, and 32.
5. The bispecific CAR of claim 1, wherein the second antigen binding domain is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 19, 21, 23, 25, 27, 29, and 31.
6. The bispecific CAR of claim 1, wherein the transmembrane domain comprises CD28.

7. The bispecific CAR of claim 1, wherein the first intracellular domain is selected from the group consisting of 4-1BB, CD28, and CD3 zeta.
8. The bispecific CAR of claim 1, wherein the second intracellular domain is selected from the group consisting of 4-1BB, CD28, and CD3 zeta.
9. The bispecific CAR of claim 1, wherein the bispecific CAR further comprises a hinge domain selected from the group consisting of a CD8 hinge, an IgG3s hinge, and an IgG4m hinge.
10. The bispecific CAR of claim 1, wherein the bispecific CAR is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NOs: 33-39.
11. A modified T cell or precursor thereof, comprising the bispecific CAR of claim 1.
12. The cell of claim 11, wherein the T cell is autologous.
13. A nucleic acid encoding the bispecific CAR of claim 1.
14. A method for treating cancer in a subject in need thereof, the method comprising administering to the subject a modified T cell or precursor thereof comprising the bispecific CAR of claim 1.
15. The method of claim 14, wherein the cancer is acute myeloid leukemia (AML).

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