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(54) **Title:** CHIMERIC ANTIGEN RECEPTOR T CELLS TARGETING THE TUMOR MICROENVIRONMENT

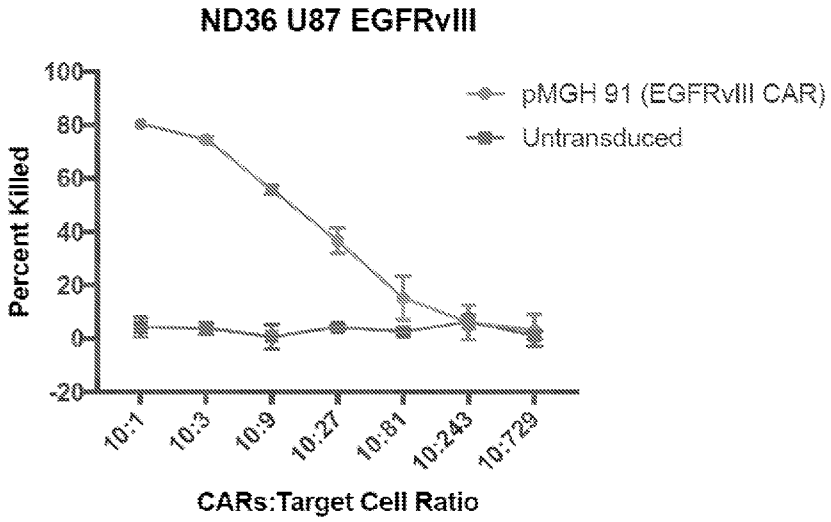

FIG. 1

(57) **Abstract:** The invention provides methods and compositions for use in treating cancer, which advantageously may be achieved by targeting of the tumor microenvironment.

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CHIMERIC ANTIGEN RECEPTOR T CELLS TARGETING THE TUMOR MICROENVIRONMENT

TECHNICAL FIELD

[0001] The technology described herein relates to immunotherapy.

BACKGROUND

[0002] Chimeric antigen receptor (CARs) provide a way to direct a cytotoxic T cell response to target cells expressing a selected target antigen, most often a tumor antigen or tumor-associated antigen. CARs are an adaptation of the T cell receptor, where the antigen binding domain is replaced with the antigen binding domain of an antibody that specifically binds the derived target antigen. Engagement of the target antigen on the surface of a target cell by a CAR expressed on a T cell ("CAR T cell" or "CAR-T") promotes killing of the target cell.

SUMMARY

[0003] The invention provides chimeric antigen receptor (CAR) T cells including a heterologous nucleic acid molecule, wherein the heterologous nucleic acid molecule includes: (a) a first polynucleotide encoding a CAR including an antigen-binding domain, a transmembrane domain, and an intracellular signaling domain; and (b) a second polynucleotide encoding a therapeutic agent. Optionally, the first and second polynucleotides are included within a single polynucleotide molecule. Furthermore, in some embodiments, the CAR further includes one or more co-stimulatory domains (e.g., 4-1BB; also see below).

[0004] In various embodiments, the therapeutic agent is or includes an antibody reagent (e.g., a single chain antibody, a single domain antibody (e.g., a camelid antibody), or a bispecific antibody reagent (e.g., a bispecific T cell engager (BiTE); also see below). In other embodiments, the therapeutic agent is or includes a cytokine.

[0005] In various embodiments, the CAR and the therapeutic agent are produced in the form of a polyprotein (and thus may be encoded within a single nucleic acid molecule), which is cleaved to generate separate CAR and therapeutic agent molecules. In some embodiments, the polyprotein includes a cleavable moiety (e.g., a 2A peptide, such as P2A or T2A; also see below) between the CAR and the therapeutic agent. In some embodiments, the CAR and the therapeutic agent are each constitutively expressed. In some embodiments, expression of the CAR and the therapeutic agent is driven by an elongation factor-1 alpha (EF1α) promoter. In some embodiments, the therapeutic agent is expressed under the control of an inducible promoter (e.g., the NFAT promoter), which is optionally inducible by T cell receptor or CAR signaling. In some embodiments, the CAR is expressed under the control of a constitutive promoter and the therapeutic agent is expressed under the control of an inducible promoter (e.g., the NFAT promoter), which is optionally inducible by T cell receptor or CAR signaling.

[0006] In various embodiments, the antigen-binding domain of the CAR is or includes an antibody, a single chain antibody, a single domain antibody (e.g., a camelid antibody), or a ligand.

[0007] In various embodiments, the transmembrane domain of the CAR includes a CD8 hinge/transmembrane domain, which optionally includes the sequence of any one of SEQ ID NOs: 4, 10, 16, 22, 28, 37, 46, 58, and 66, or a variant thereof.

[0008] In various embodiments, the intracellular signaling domain includes a CD3ζ intracellular signaling domain, which optionally includes or consists of the sequence of any one of SEQ ID NOs: 6, 12, 18, 24, 30, 39, 48, 60, and 68, or a variant thereof.

[0009] In various embodiments, a 4-1BB co-stimulatory domain is included, which optionally includes or consists of the sequence of any one of SEQ ID NOs: 5, 11, 17, 23, 29, 38, 47, 59, and 67, or a variant thereof.

[0010] In various embodiments, the CAR antigen-binding domain or the therapeutic agent, when the therapeutic agent is or includes an antibody reagent, bind to a tumor-associated antigen (see, e.g., below). In various embodiments, the tumor-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds is a solid tumor-associated antigen. In various embodiments, the tumor-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds includes epidermal growth factor receptor variant III (EGFRvIII), EGFR, CD19, prostate-specific membrane antigen (PSMA), or IL-13 receptor alpha 2 (IL-13Rα2), and optionally the CAR antigen-binding domain or the therapeutic agent includes a sequence selected from the group consisting of SEQ ID NO: 21, 27, 33, 36, 42, 45, 51, 55, 57, 63, 65, and variants thereof.

[0011] In various embodiments, the CAR antigen-binding domain or the therapeutic agent, when the therapeutic agent is or includes an antibody reagent, binds to a Treg-associated antigen. In various embodiments, the Treg-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds is selected from the group consisting of glycoprotein A repetitions predominant (GARP), latency-associated peptide (LAP), CD25, and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and optionally the CAR antigen-binding domain or the therapeutic agent includes a sequence selected from the group consisting of SEQ ID NO: 3, 9, 15, 25, and variants thereof.

[0012] The invention further provides CAR T cells including a polynucleotide encoding a CAR, wherein the CAR includes an antigen-binding domain, a transmembrane domain (e.g., CD8 hinge/TM; see, e.g., below for additional examples), and an intracellular signaling domain (e.g., CD3z; see, e.g., below for additional examples); and the antigen-binding domain binds to a Treg-associated antigen. In various embodiments, the Treg-associated antigen is selected from the group consisting of GARP, LAP, CD25, and CTLA-4. In various embodiments, the CAR further includes one or more co-stimulatory domains (e.g., 4-1BB; see, e.g., below, for additional examples). In various examples, the antigen-binding domain of the CAR includes a scFv or a single domain antibody, which optionally includes a sequence selected from the group consisting of SEQ ID NO: 3, 9, 15, 25, and variants thereof.

[0013] The invention further provides CAR T cells including a heterologous nucleic acid molecule encoding an amino acid sequence having at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or 100%) sequence identity to the amino acid sequence of any one of SEQ ID NO: 26, SEQ ID NO: 35, SEQ ID NO: 44, SEQ ID NO: 53, SEQ ID NO: 61, SEQ ID NO: 19, SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13.

[0014] Further, the invention provides nucleic acid molecules encoding (i) CAR polypeptides or (ii) polyproteins including a CAR polypeptide and a therapeutic agent, as described herein.

[0015] The invention additional provides (i) CAR polypeptides or (ii) polyproteins including a CAR polypeptide and a therapeutic agent, as described herein.

[0016] The invention additional provides pharmaceutical compositions including one or more CAR T cells, nucleic acid molecules, CAR polypeptides, or a polyproteins as described herein.

[0017] Also included in the invention are methods of treating subject, including patients (e.g., human patients) having a disease or condition (e.g., cancer (such as glioblastoma); also see below). The methods include administering to the subject a pharmaceutical composition including one or more CAR T cells or pharmaceutical compositions described herein. In various embodiments, the methods target the tumor microenvironment, by which, e.g., systemic toxicity is reduced. In various embodiments, the cancer is characterized by the presence of one or more solid tumors. In various embodiments, the cancer is characterized by tumor-infiltrating Tregs.

[0018] The invention further provides methods of treating a subject (e.g., a patient, such as a human patient) having cancer. The methods include administering to the subject a CAR T cell product, genetically modified to secrete a tumor-toxic antibody or cytokine, wherein by directing the cancer toxicity locally to the tumor microenvironment, systemic toxicity is reduced. In various embodiments, the CAR T cell is genetically modified to deliver an antibody against CTLA4, CD25, GARP, LAP, IL15, CSF1R, or EGFR, or a bispecific antibody (directed against, e.g., EGFR and CD3) against to the tumor microenvironment.

[0019] The invention additionally provides methods of delivering one or more therapeutic agents to a tissue or organ in a patient to treat a disease or pathology. The methods include administering to the patient a CAR T cell, genetically modified to secrete a therapeutic antibody, toxin, or agent, wherein the therapeutic antibody, toxin, or agent would, by itself, be unable to enter or penetrate the tissue or organ. In various embodiments, the tissue or organ is in the nervous system. In various embodiments, the nervous system is the central nervous system (e.g., brain). In various embodiments, the disease or pathology is glioblastoma. In various embodiments, the therapeutic antibody is anti-EGFR (anti-epidermal growth factor receptor) or anti-EGFRvIII.

[0020] The invention also includes use of the CAR T cells, polypeptides, nucleic acid molecules, pharmaceutical compositions, and other compositions and molecules in the use of preventing or treating a disease or condition described herein, or in the use of the preparation of a medicament therefor.

[0021] **Definitions**

[0022] For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed technology, because the scope of the technology is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this technology belongs. If there is an apparent discrepancy between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[0023] Definitions of common terms in immunology and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 19th Edition, published by Merck Sharp & Dohme Corp., 2011 (ISBN 978-0-911910-19-3); Robert S. Porter et al. (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by

VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), Taylor & Francis Limited, 2014 (ISBN 0815345305, 9780815345305); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737), the contents of each of which are all incorporated by reference herein in their entireties.

[0024] The terms "decrease," "reduced," "reduction," or "inhibit" are all used herein to mean a decrease by a statistically significant amount. In some embodiments, "reduce," "reduction," or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g., the absence of a given treatment or agent) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, "reduction" or "inhibition" does not encompass a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition as compared to a reference level. Where applicable, a decrease can be preferably down to a level accepted as within the range of normal for an individual without a given disorder.

[0025] The terms "increased," "increase," "enhance," or "activate" are all used herein to mean an increase by a statically significant amount. In some embodiments, the terms "increased," "increase," "enhance," or "activate" can mean an increase of at least 10% as compared to a reference level, for example, an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, an "increase" is a statistically significant increase in such level.

[0026] As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include, for example, chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., rhesus. Rodents include, for example, mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include, for example, cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In

some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, "individual," "patient," and "subject" are used interchangeably herein.

[0027] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of disease, e.g., cancer. A subject can be male or female.

[0028] A subject can be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment (e.g., glioblastoma, glioma, leukemia, or another type of cancer, among others) or one or more complications related to such a condition, and optionally, have already undergone treatment for the condition or the one or more complications related to the condition. Alternatively, a subject can also be one who has not been previously diagnosed as having such condition or related complications. For example, a subject can be one who exhibits one or more risk factors for the condition or one or more complications related to the condition or a subject who does not exhibit risk factors.

[0029] A "subject in need" of treatment for a particular condition can be a subject having that condition, diagnosed as having that condition, or at risk of developing that condition.

[0030] A "disease" is a state of health of an animal, for example, a human, 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.

[0031] As used herein, the terms "tumor antigen" and "cancer antigen" are used interchangeably to refer to antigens that are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens that can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), and fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses. Many tumor antigens have been defined in terms of multiple solid tumors: MAGE 1, 2, & 3, defined by immunity; MART-1/Melan-A, gp100, carcinoembryonic antigen (CEA), HER2, mucins (i.e., MUC-1), prostate-specific antigen (PSA), and prostatic acid phosphatase (PAP). In addition, viral proteins such as some encoded by hepatitis B (HBV), Epstein-Barr (EBV), and human papilloma (HPV) have been shown to be important in the development of hepatocellular carcinoma, lymphoma, and cervical cancer, respectively. Examples of tumor antigens are provided below and include, e.g., EGFR, EGFRvIII, CD19, PSMA, BCMA, IL13Ra2, etc.

[0032] As used herein, "Treg antigen" or "Treg-associated antigen" are used interchangeably to refer to antigens that are expressed by T regulatory (Treg) cells. These antigens may optionally be targeted by

the cells and methods of the invention. Examples of Treg antigens are provided below and include, e.g., GARP, LAP, CD25, and CTLA4.

[0033] As used herein, the term "chimeric" refers to the product of the fusion of portions of at least two or more different polynucleotide molecules. In one embodiment, the term "chimeric" refers to a gene expression element produced through the manipulation of known elements or other polynucleotide molecules.

[0034] By "bi-specific T cell engagers," "BiTE antibody constructs," or BiTEs" is meant polypeptides that each include tandemly linked single-chain variable fragments (scFvs). Optionally, the scFvs are linked by a linker (e.g., a glycine-rich linker). One scFv of the BiTE binds to the T cell receptor (TCR) (e.g., to the CD3ε subunit) and the other binds to a target antigen (e.g., a tumor-associated antigen).

[0035] In some embodiments, "activation" can refer to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. In some embodiments activation can refer to induced cytokine production. In other embodiments, activation can refer to detectable effector functions. At a minimum, an "activated T cell" as used herein is a proliferative T cell.

[0036] As used herein, the terms "specific binding" and "specifically binds" refer to a physical interaction between two molecules, compounds, cells and/or particles wherein the first entity binds to the second, target, entity with greater specificity and affinity than it binds to a third entity which is a non-target. In some embodiments, specific binding can refer to an affinity of the first entity for the second target, entity, which is at least 10 times, at least 50 times, at least 100 times, at least 500 times, at least 1000 times or more greater than the affinity for the third non-target entity under the same conditions. A reagent specific for a given target is one that exhibits specific binding for that target under the conditions of the assay being utilized. A non-limiting example includes an antibody, or a ligand, which recognizes and binds with a cognate binding partner (for example, a stimulatory and/or costimulatory molecule present on a T cell) protein.

[0037] A "stimulatory ligand," as used herein, refers to a ligand that when present on an antigen presenting cell (APC, e.g., a macrophage, a dendritic cell, a B-cell, an artificial APC, and the like) can specifically bind with a cognate binding partner (referred to herein as a "stimulatory molecule" or "co-stimulatory molecule") on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, proliferation, activation, initiation of an immune response, and the like. Stimulatory ligands are well-known in the art and encompass, inter alia, an MHC Class I molecule loaded with a peptide, an anti-CD3 antibody, a superagonist anti-CD28 antibody, and a superagonist anti-CD2 antibody.

[0038] 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.

[0039] "Co-stimulatory ligand," as the term is used herein, includes a molecule on an APC 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, 4-1BBL, OX40L, CD7, B7-1 (CD80), B7-2 (CD86), PD-L1, PD-L2, 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-like receptor

and a ligand that specifically binds with B7-H3. A co-stimulatory ligand also can include, but is not limited to, 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.

[0040] 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, a Toll-like receptor, CD27, CD28, 4-1BB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and CD83.

[0041] In one embodiment, the term "engineered" and its grammatical equivalents as used herein can refer to one or more human-designed alterations of a nucleic acid, e.g., the nucleic acid within an organism's genome. In another embodiment, engineered can refer to alterations, additions, and/or deletion of genes. An "engineered cell" can refer to a cell with an added, deleted and/or altered gene. The term "cell" or "engineered cell" and their grammatical equivalents as used herein can refer to a cell of human or non-human animal origin.

[0042] As used herein, the term "operably linked" refers to a first polynucleotide molecule, such as a promoter, connected with a second transcribable polynucleotide molecule, such as a gene of interest, where the polynucleotide molecules are so arranged that the first polynucleotide molecule affects the function of the second polynucleotide molecule. The two polynucleotide molecules may or may not be part of a single contiguous polynucleotide molecule and may or may not be adjacent. For example, a promoter is operably linked to a gene of interest if the promoter regulates or mediates transcription of the gene of interest in a cell.

[0043] In the various embodiments described herein, it is further contemplated that variants (naturally occurring or otherwise), alleles, homologs, conservatively modified variants, and/or conservative substitution variants of any of the particular polypeptides described are encompassed. As to amino acid sequences, one of ordinary skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid and retains the desired activity of the polypeptide. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles consistent with the disclosure.

[0044] A given amino acid can be replaced by a residue having similar physiochemical characteristics, e.g., substituting one aliphatic residue for another (such as Ile, Val, Leu, or Ala for one another), or substitution of one polar residue for another (such as between Lys and Arg; Glu and Asp; or Gln and Asn). Other such conservative substitutions, e.g., substitutions of entire regions having similar hydrophobicity characteristics, are well known. Polypeptides comprising conservative amino acid substitutions can be tested in any one of the assays described herein to confirm that a desired activity, e.g., ligand-mediated receptor activity and specificity of a native or reference polypeptide is retained.

[0045] Amino acids can be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: Gly (G), Ser

(S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q); (3) acidic: Asp (D), Glu (E); (4) basic: Lys (K), Arg (R), His (H). Alternatively, naturally occurring residues can be divided into groups based on common side-chain properties: (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; (6) aromatic: Trp, Tyr, Phe. Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Particular conservative substitutions include, for example; Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

[0046] In some embodiments, a polypeptide described herein (or a nucleic acid encoding such a polypeptide) can be a functional fragment of one of the amino acid sequences described herein. As used herein, a "functional fragment" is a fragment or segment of a peptide that retains at least 50% of the wildtype reference polypeptide's activity according to an assay known in the art or described below herein. A functional fragment can comprise conservative substitutions of the sequences disclosed herein.

[0047] In some embodiments, a polypeptide described herein can be a variant of a polypeptide or molecule as described herein. In some embodiments, the variant is a conservatively modified variant. Conservative substitution variants can be obtained by mutations of native nucleotide sequences, for example. A "variant," as referred to herein, is a polypeptide substantially homologous to a native or reference polypeptide, but which has an amino acid sequence different from that of the native or reference polypeptide because of one or a plurality of deletions, insertions, or substitutions. Variant polypeptide-encoding DNA sequences encompass sequences that comprise one or more additions, deletions, or substitutions of nucleotides when compared to a native or reference DNA sequence, but that encode a variant protein or fragment thereof that retains activity of the non-variant polypeptide. A wide variety of PCR-based site-specific mutagenesis approaches are known in the art and can be applied by the ordinarily skilled artisan.

[0048] A variant amino acid or DNA sequence can be at least 80%, at least 85%, 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 more, identical to a native or reference sequence. The degree of homology (percent identity) between a native and a mutant sequence can be determined, for example, by comparing the two sequences using freely available computer programs commonly employed for this purpose on the world wide web (e.g., BLASTp or BLASTn with default settings).

[0049] Alterations of the native amino acid sequence can be accomplished by any of a number of techniques known to one of skill in the art. Mutations can be introduced, for example, at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites permitting ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion. Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered nucleotide sequence having particular codons altered according to the substitution, deletion, or insertion required. Techniques for making such alterations are well established and include, for example, those disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985,

12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462, which are herein incorporated by reference in their entireties. Any cysteine residue not involved in maintaining the proper conformation of a polypeptide also can be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) can be added to a polypeptide to improve its stability or facilitate oligomerization.

[0050] As used herein, the term "DNA" is defined as deoxyribonucleic acid. The term "polynucleotide" is used herein interchangeably with "nucleic acid" to indicate a polymer of nucleosides. Typically a polynucleotide is composed of nucleosides that are naturally found in DNA or RNA (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine) joined by phosphodiester bonds. However, the term encompasses molecules comprising nucleosides or nucleoside analogs containing chemically or biologically modified bases, modified backbones, etc., whether or not found in naturally occurring nucleic acids, and such molecules may be preferred for certain applications. Where this application refers to a polynucleotide it is understood that both DNA, RNA, and in each case both single- and double-stranded forms (and complements of each single-stranded molecule) are provided. "Polynucleotide sequence" as used herein can refer to the polynucleotide material itself and/or to the sequence information (i.e., the succession of letters used as abbreviations for bases) that biochemically characterizes a specific nucleic acid. A polynucleotide sequence presented herein is presented in a 5' to 3' direction unless otherwise indicated.

[0051] The term "polypeptide" as used herein refers to a polymer of amino acids. The terms "protein" and "polypeptide" are used interchangeably herein. A peptide is a relatively short polypeptide, typically between about 2 and 60 amino acids in length. Polypeptides used herein typically contain amino acids such as the 20 L-amino acids that are most commonly found in proteins. However, other amino acids and/or amino acid analogs known in the art can be used. One or more of the amino acids in a polypeptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a fatty acid group, a linker for conjugation, functionalization, etc. A polypeptide that has a nonpolypeptide moiety covalently or noncovalently associated therewith is still considered a "polypeptide." Exemplary modifications include glycosylation and palmitoylation. Polypeptides can be purified from natural sources, produced using recombinant DNA technology or synthesized through chemical means such as conventional solid phase peptide synthesis, etc. The term "polypeptide sequence" or "amino acid sequence" as used herein can refer to the polypeptide material itself and/or to the sequence information (i.e., the succession of letters or three letter codes used as abbreviations for amino acid names) that biochemically characterizes a polypeptide. A polypeptide sequence presented herein is presented in an N-terminal to C-terminal direction unless otherwise indicated.

[0052] In some embodiments, a nucleic acid encoding a polypeptide as described herein (e.g., a CAR polypeptide) is comprised by a vector. In some of the aspects described herein, a nucleic acid sequence encoding a given polypeptide as described herein, or any module thereof, is operably linked to a vector. The term "vector," as used herein, refers to a nucleic acid construct designed for delivery to a host cell or for transfer between different host cells. As used herein, a vector can be viral or non-viral. The term "vector" encompasses any genetic element that is capable of replication when associated with

the proper control elements and that can transfer gene sequences to cells. A vector can include, but is not limited to, a cloning vector, an expression vector, a plasmid, phage, transposon, cosmid, artificial chromosome, virus, virion, etc.

[0053] As used herein, the term "expression vector" refers to a vector that directs expression of an RNA or polypeptide from sequences linked to transcriptional regulatory sequences on the vector. The sequences expressed will often, but not necessarily, be heterologous to the cell. An expression vector may comprise additional elements, for example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example, in human cells for expression and in a prokaryotic host for cloning and amplification. The term "expression" refers to the cellular processes involved in producing RNA and proteins and as appropriate, secreting proteins, including where applicable, but not limited to, for example, transcription, transcript processing, translation and protein folding, modification and processing. "Expression products" include RNA transcribed from a gene, and polypeptides obtained by translation of mRNA transcribed from a gene. The term "gene" means the nucleic acid sequence which is transcribed (DNA) to RNA *in vitro* or *in vivo* when operably linked to appropriate regulatory sequences. The gene may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5'UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

[0054] As used herein, the term "viral vector" refers to a nucleic acid vector construct that includes at least one element of viral origin and has the capacity to be packaged into a viral vector particle. The viral vector can contain a nucleic acid encoding a polypeptide as described herein in place of non-essential viral genes. The vector and/or particle may be utilized for the purpose of transferring nucleic acids into cells either *in vitro* or *in vivo*. Numerous forms of viral vectors are known in the art.

[0055] By "recombinant vector" is meant a vector that includes a heterologous nucleic acid sequence or "transgene" that is capable of expression *in vivo*. It should be understood that the vectors described herein can, in some embodiments, be combined with other suitable compositions and therapies. In some embodiments, the vector is episomal. The use of a suitable episomal vector provides a means of maintaining the nucleotide of interest in the subject in high copy number extra-chromosomal DNA thereby eliminating potential effects of chromosomal integration.

[0056] As used herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down, or stop the progression or severity of a condition associated with a disease or disorder, e.g., glioblastoma, glioma, acute lymphoblastic leukemia or other cancer, disease, or disorder. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder. Treatment is generally "effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or

decreased mortality, whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side effects of the disease (including palliative treatment).

[0057] As used herein, the term "pharmaceutical composition" refers to the active agent in combination with a pharmaceutically acceptable carrier e.g. a carrier commonly used in the pharmaceutical industry. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. In some embodiments of any of the aspects, a pharmaceutically acceptable carrier can be a carrier other than water. In some embodiments of any of the aspects, a pharmaceutically acceptable carrier can be a cream, emulsion, gel, liposome, nanoparticle, and/or ointment. In some embodiments of any of the aspects, a pharmaceutically acceptable carrier can be an artificial or engineered carrier, e.g., a carrier in which the active ingredient would not be found to occur in nature.

[0058] As used herein, the term "administering," refers to the placement of a therapeutic or pharmaceutical composition as disclosed herein into a subject by a method or route that results in at least partial delivery of the agent at a desired site. Pharmaceutical compositions comprising agents as disclosed herein can be administered by any appropriate route that results in an effective treatment in the subject.

[0059] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[0060] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[0061] As used herein, the term "comprising" means that other elements can also be present in addition to the defined elements presented. The use of "comprising" indicates inclusion rather than limitation.

[0062] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0063] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the technology.

[0064] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[0065] In some embodiments of any of the aspects, the disclosure described herein does not concern a process for cloning human beings, processes for modifying the germ line genetic identity of human beings, uses of human embryos for industrial or commercial purposes or processes for modifying

the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes.

[0066] Other terms are defined within the description of the various aspects and embodiments of the technology, as set forth below.

[0067] The invention provide several advantages. For example, the CAR T cells of the invention can be used to deliver therapeutic molecules for cancer treatment. In one example, the CAR T cells of the invention can be used to deliver otherwise toxic antibodies (e.g., anti-CTLA4 or anti-CD25 (e.g., daclizumab) or other molecules (e.g., cytokines) to the tumor microenvironment, where they can advantageously enable activation of surrounding tumor infiltrating lymphocytes, provide checkpoint blockade, and deplete regulatory T cells (Tregs). The CAR T cells of the invention can further be directed against Treg antigens, to facilitate targeting of Treg cells. Furthermore, certain CAR T cells of the invention can be used to deliver genetically encoded molecules (e.g., antibodies or cytokines) to regions of the body (e.g., the central nervous system, including the brain) that these molecules otherwise cannot reach. In one example, CAR T cells targeting EGFRvIII can be used to target brain tumors, and can deliver antibodies (e.g., antibodies against EGFR, such as cetuximab; also see below) to the tumors. The invention thus provides genetically-encoded Treg targeting in the tumor microenvironment. In addition, the invention provides genetically-encoded delivery of antibodies that cannot get into certain tissues, and could enhance the potency of T cell therapies by broadening the specificity of the anti-tumor target. The invention accordingly provides for gene-modified T cell therapy for cancer.

[0068] Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0069] Fig. 1 is a graph showing killing of human glioma target cell line U87vIII by CART-EGFRvIII cells as a function of CART-EGFRvIII:U87vIII target cell ratio. Untransduced cells were incubated with target cells as a negative control.

[0070] Figs. 2A and 2B are a series of x-ray overlays showing the location of EGFRvIII expressing tumor (U87vIII) in a subcutaneous model of human glioma. Fig. 2A shows mice treated with untransduced cells as a negative control. Fig. 2B shows mice treated with CART-EGFRvIII on day 4 after implantation (top row), with successful treatment by day 21 (bottom row).

[0071] Figs. 3A and 3B are a series of x-ray overlays showing the location of EGFRvIII expressing tumor (U87vIII) in a intracranial model of human glioma. Fig. 2A shows mice treated with untransduced (UTD) cells as a negative control at day 5 (D5; top) and D11 (bottom). Fig. 2B shows mice treated with CART-EGFRvIII on day 2 after implantation at D5 (top row) and at D21 (bottom row).

[0072] Figs. 4A and 4B are photomicrographs showing immunohistochemistry of tumor tissue in one patient five days following infusion of CART-EGFRvIII. Fig. 4A shows T cells stained for CD3. Fig. 4B shows CD25+ cells. CD25 is the IL-2 receptor alpha chain, a marker of activated or regulatory T cells.

[0073] Figs. 5A-5C are fluorescence micrographs qualitatively demonstrating Treg suppression of CAR T cell (red) antitumor activity after 18 hours of coincubation with human glioma cells (green) in vitro. Fig. 5A shows relative concentration of CART-nonspecific cells to glioma cells. Fig. 5B shows relative concentration of CART-EGFRvIII cells to glioma cells with no Tregs in the culture. Fig. 5C shows relative concentration of CART-EGFRvIII cells to glioma cells with Tregs included in the culture.

[0074] Fig. 5D is a graph showing quantitative readouts of green object confluence as a measure of glioma cell viability as a function of time (up to 48 hours). The top line represents the results shown in Fig. 5A (glioma cell growth), the bottom line represents the results shown in Fig. 5B (glioma cell killing), and the middle line represents the results shown in Fig. 5C (glioma cell resistance to CART-killing).

[0075] Figs. 6A-6C are flow cytometry plots showing expression of LAP (x-axis) and GARP (y-axis) on control T cells (Fig. 6A), unactivated Tregs (Fig. 6B), and activated Tregs (Fig. 6C). Tregs were sorted from leukopak on CD4+CD25+CD127- and expanded with CD3/CD28 beads for seven days in the presence of IL-2. On day 1, they were transduced to expressed GFP. After debeading on day 7, expanded Tregs were rested for four days before freezing. After thawing, Tregs were stained for LAP and GARP expression after overnight rest (non-activated) or overnight activation with anti-CD3 and anti-CD28. Untransduced T cells (CD4+ and CD8+) from the same donor were used as controls for expression (Fig. 6A).

[0076] Figs. 7A and 7B are flow cytometry histograms corresponding to the results shown in Figs. 6A-6C showing expression of LAP (Fig. 7A) and GARP (Fig. 7B).

[0077] Figs. 8A-8D are schematic drawings of CAR constructs for targeting Treg-associated antigens. Fig. 8A shows a LAP-targeting CAR construct having an anti-LAP scFv with its light chain (L) and heavy chain (H) arranged in a 5'-to-3' direction, respectively (CART-LAP-L-H). Fig. 8B shows a LAP-targeting CAR construct having an anti-LAP scFv with its heavy chain (H) and light chain (L) arranged in a 5'-to-3' direction, respectively (CART-LAP-H-L). Fig. 8C shows a GARP-targeting CAR construct having an anti-GARP camelid antibody binding domain (CART-GARP). Fig. 8D shows an EGFR-targeted CAR construct having an anti-GARP camelid antibody.

[0078] Figs. 9A and 9B are graphs showing target Treg killing as a function of CAR T cell-to-target Treg cell ratio. Tregs were transduced with GFP, and cytotoxicity was quantified by monitoring GFP expression. Fig. 9A shows killing of activated Tregs, and Fig. 9B shows killing of non-activated Tregs. CART-LAP-H-L was more effective at killing non-activated Tregs in comparison to CART-LAP-L-H.

[0079] Figs. 10A and 10B are graphs showing target Treg killing by various anti-Treg CAR T cells (i.e., CART-GARP, CART-LAP-H-L, CART-LAP-L-H, or untransduced control cells) at a 1:1 ratio of CAR T cells to Tregs for four days. Fig. 10A and 10B show results from the same experiment conducted in two different donors.

[0080] Figs. 11A-11D are graphs showing target Treg killing as a function of CAR T cell-to-target Treg cell ratio by LAP-targeted CAR T cells after three days of coculture. Figs. 11A and 11B show number of target cells remaining in coculture as measured by flow cytometry. A dashed line indicates the number of target cells in a control sample containing no CAR cells. Fig. 11A shows non-activated Tregs as target cells, whereas Fig. 11B shows activated Tregs as target cells. Figs. 11C and 11D show percent cytotoxicity as measured by luciferase expression by target cells. Fig. 11C shows non-activated Tregs as target cells, whereas Fig. 11D shows activated Tregs as target cells. In each of 11A-11D, circles represent CART-LAP-H-L, squares represent CART-LAP-L-H, and triangles represent untransduced CAR cells.

[0081] Figs. 12A and 12B are flow cytometry histograms showing the expression of GARP (Fig. 12A) and LAP (Fig. 12B) by HUT78 cells.

[0082] Figs. 13A and 13B are graphs showing killing of target HUT78 cells as a function of CAR T cell-to-target cell ratio by LAP-targeted CAR T cells after three days of coculture. Fig. 13A shows the number of target cells remaining in culture after three days, as measured by flow cytometry. A dashed line indicates the number of target cells in a control sample containing no CAR cells. Fig. 13B shows percent cytotoxicity as measured by luciferase expression by target cells. Circles represent CART-LAP-H-L, squares represent CART-LAP-L-H, and triangles represent untransduced CAR cells.

[0083] Figs. 14A and 14B are flow cytometry histograms showing the expression of GARP (Fig. 14A) and LAP (Fig. 14B) by SeAx cells.

[0084] Figs. 15A and 15B are graphs showing killing of target SeAx cells as a function of CAR T cell-to-target cell ratio by GARP and LAP-targeted CAR T cells after 24 (Fig. 15A) hours and 48 hours (Fig. 15B) of coculture, as measured by luciferase expression by target cells. Squares represent CART-GARP, upward-facing triangles represent CART-LAP-H-L, downward-facing triangles represent CART-LAP-H-L cells, and diamonds represent untransduced CAR cells.

[0085] Figs. 16A-16C are photographs of western blots showing the presence of protein components of supernatants obtained from cultures of CART-EGFR-GARP T cells. Fig. 16A and 16B show the full gel, including molecular weight reference ladders. Fig. 16C is a longer exposure of the bottom region of the gel shown in Fig. 16B, in which a band between 10 and 15 kD is identified with an arrow, indicating the presence of a camelid antibody.

[0086] Fig. 17 is a schematic drawing of CAR-EGFR-BiTE-(EGFR-CD3), an exemplary nucleic acid molecule encoding a CAR and a BiTE.

[0087] Fig. 18 is a schematic drawing of a BiTE having an anti-EGFR domain derived from cetuximab and an anti-CD3 domain derived from blinatumomab.

[0088] Fig. 19 is a set of photographs showing a western blot experiment verifying the presence of BiTE in lane 2.

[0089] Figs. 20A and 20B are a set of flow cytometry graphs showing binding of BiTE expressed by HEK293 cells transduced with CAR-EGFR-BiTE-(EGFR-CD3) to EGFR expressed by K562 cells (Fig. 20A) and CD3 expressed by Jurkat cells (Fig. 20B).

[0090] Figs. 21A and 21B are a set of flow cytometry graphs showing binding of BiTE expressed by SupT1 cells transduced with CAR-EGFR-BiTE-(EGFR-CD3) to EGFR expressed by K562 cells (Fig. 21A) and CD3 expressed by CAR-EGFR-BiTE-(EGFR-CD3)-expressing SupT1 cells (Fig. 21B).

[0091] Figs. 22A and 22B are a set of flow cytometry graphs showing binding of BiTE expressed by ND4 cells transduced with CAR-EGFR-BiTE-(EGFR-CD3) to EGFR expressed by K562 cells (Fig. 21A) and CD3 expressed by CAR-EGFR-BiTE-(EGFR-CD3)-expressing ND4 cells (Fig. 21B).

[0092] Fig. 23 is a graph showing killing of U87vIII cells by ND4 cells incubated with BiTE secreted by HEK293T cells that were transduced with CAR-EGFR-BiTE-(EGFR-CD3), as a function of effector (untransduced ND4) to target (U87vIII) cell ratio. Squares represent the experimental group in which the supernatant contained BiTE, and circles represent a negative control containing no BiTE.

[0093] Fig. 24 is a drawing of an exemplary nucleic acid molecule encoding a CAR under control of an EF1α promoter and GFP under control of an NFAT promoter.

[0094] Figs. 25A and 25B are a set of flow cytometry graphs showing GFP expression by cells transduced with the construct of Fig. 24. The red histogram shows GFP expression in unstimulated cells; the blue histogram shows GFP expression in cells stimulated with PMA and ionomycin; and the orange histogram shows GFP expression in cells coated with PEPvIII.

[0095] Fig. 26A is a schematic drawing of GFP-CAR-EGFR-BiTE-(EGFR-CD3), an exemplary nucleic acid molecule encoding a CAR and a constitutively expressed BiTE.

[0096] Fig. 26B is a schematic drawing of GFP-CAR-EGFR-BiTE-(CD19-CD3), an exemplary nucleic acid molecule encoding a CAR and a constitutively expressed BiTE.

[0097] Fig. 27A is a schematic drawing of BiTE-(CD19-CD3)-CAR-EGFR, an exemplary nucleic acid molecule encoding a CAR and an inducibly expressed BiTE.

[0098] Fig. 27B is a schematic drawing of BiTE-(CD19-CD3)-CAR-EGFR, an exemplary nucleic acid molecule encoding a CAR and an inducibly expressed BiTE.

DETAILED DESCRIPTION

[0099] The invention provides improved approaches to chimeric antigen receptor T cell ("CAR T cell")-based therapy. In general, the improvements relate to different aspects of targeting in antitumor therapy, for example, targeting of the tumor microenvironment.

[00100] As is explained further below, we have demonstrated that regulatory T cells (also referred to herein as "Tregs"), which play a role in the suppression of a subject's immune response against tumors (e.g., in the tumor microenvironment), can be targeted with CAR T cells. The invention thus provides CAR T cells, in which the CAR is directed against a Treg antigen or marker (e.g., GARP, LAP, CTLA4, or CD25; also see below). In other examples, the invention provides CAR T cells that secrete antibodies (e.g., single chain antibodies, single domain antibodies (e.g., camelid antibodies), or bispecific antibodies (e.g., bispecific T cell engagers)) against one or more Treg antigens or markers (e.g., GARP, LAP, CTLA4 and CD25; also see below). In addition to targeting Tregs, the invention provides CAR T cells and related methods for delivering other therapeutic agents (e.g., antibodies and related molecules) to tumors. In one example, a CAR T cell having a CAR specific for EGFRvIII is used to target brain tumors (e.g., glioblastomas). Such CAR T cells may also be used to deliver therapeutic molecules, such as antibody reagents (e.g., single chain antibodies, single domain antibodies (e.g., camelid antibodies), or bi-specific antibodies (e.g., bispecific T cell engagers)) to these tumors. These methods are particularly advantageous, as they, in effect, facilitate antibody administration to the brain, despite the blood brain barrier through which antibodies do not normally pass. These approaches, as well as related methods and compositions, are described further, as follows.

[00101] **Chimeric Antigen Receptors**

[00102] The technology described herein provides improved CARs for use in immunotherapy. The following discusses CARs and the various improvements.

[00103] The terms "chimeric antigen receptor" or "CAR" or "CARs" as used herein refer to engineered T cell receptors, which graft a ligand or antigen specificity onto T cells (for example, naïve T cells, central memory T cells, effector memory T cells or combinations thereof). CARs are also known as artificial T-cell receptors, chimeric T-cell receptors or chimeric immunoreceptors.

[00104] A CAR places a chimeric extracellular target-binding domain that specifically binds a target, e.g., a polypeptide, expressed on the surface of a cell to be targeted for a T cell response onto a

construct including a transmembrane domain and intracellular domain(s) of a T cell receptor molecule. In one embodiment, the chimeric extracellular target-binding domain comprises the antigen-binding domain(s) of an antibody that specifically binds an antigen expressed on a cell to be targeted for a T cell response. The properties of the intracellular signaling domain(s) of the CAR can vary as known in the art and as disclosed herein, but the chimeric target/antigen-binding domains(s) render the receptor sensitive to signaling activation when the chimeric target/antigen binding domain binds the target/antigen on the surface of a targeted cell.

[00105] With respect to intracellular signaling domains, so-called "first- generation" CARs include those that solely provide CD3zeta (CD3ζ) signals upon antigen binding. So-called "second-generation" CARs include those that provide both co-stimulation (e.g., CD28 or CD 137) and activation (CD3ζ) domains, and so-called "third-generation" CARs include those that provide multiple costimulatory (e.g., CD28 and CD 137) domains and activation domains (e.g., CD3ζ). In various embodiments, the CAR is selected to have high affinity or avidity for the target/antigen – for example, antibody-derived target or antigen binding domains will generally have higher affinity and/or avidity for the target antigen than would a naturally-occurring T cell receptor. This property, combined with the high specificity one can select for an antibody provides highly specific T cell targeting by CAR T cells.

[00106] As used herein, a "CAR T cell" or "CAR-T" refers to a T cell that expresses a CAR. When expressed in a T cell, CARs have the ability to redirect T-cell specificity and reactivity toward a selected target in a non-MHC-restricted manner, exploiting the antigen-binding properties of monoclonal antibodies. The non-MHC-restricted antigen recognition gives T-cells expressing CARs the ability to recognize an antigen independent of antigen processing, thus bypassing a major mechanism of tumor escape.

[00107] As used herein, the term "extracellular target binding domain" refers to a polypeptide found on the outside of the cell that is sufficient to facilitate binding to a target. The extracellular target binding domain will specifically bind to its binding partner, i.e., the target. As non-limiting examples, the extracellular target-binding domain can include an antigen-binding domain of an antibody or antibody reagent, or a ligand, which recognizes and binds with a cognate binding partner protein. In this context, a ligand is a molecule that binds specifically to a portion of a protein and/or receptor. The cognate binding partner of a ligand useful in the methods and compositions described herein can generally be found on the surface of a cell. Ligand:cognate partner binding can result in the alteration of the ligand-bearing receptor, or activate a physiological response, for example, the activation of a signaling pathway. In one embodiment, the ligand can be non-native to the genome. Optionally, the ligand has a conserved function across at least two species.

[00108] **Antibody Reagents**

[00109] In various embodiments, the CARs described herein comprise an antibody reagent or an antigen-binding domain thereof as an extracellular target-binding domain.

[00110] As used herein, the term "antibody reagent" refers to a polypeptide that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence and which specifically binds a given antigen. An antibody reagent can comprise an antibody or a polypeptide comprising an antigen-binding domain of an antibody. In some embodiments of any of the aspects, an antibody reagent can comprise a monoclonal antibody or a polypeptide comprising an antigen-binding domain of a

monoclonal antibody. For example, an antibody can include a heavy (H) chain variable region (abbreviated herein as V_H), and a light (L) chain variable region (abbreviated herein as V_L). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term "antibody reagent" encompasses antigen-binding fragments of antibodies (e.g., single chain antibodies, Fab and sFab fragments, F(ab')2, Fd fragments, Fv fragments, scFv, CDRs, and domain antibody (dAb) fragments (see, e.g., de Wildt et al., Eur. J. Immunol. 26(3):629-639, 1996; which is incorporated by reference herein in its entirety)) as well as complete antibodies. An antibody can have the structural features of IgA, IgG, IgE, IgD, or IgM (as well as subtypes and combinations thereof). Antibodies can be from any source, including mouse, rabbit, pig, rat, and primate (human and non-human primate) and primatized antibodies. Antibodies also include midibodies, humanized antibodies, chimeric antibodies, and the like. Fully human antibody binding domains can be selected, for example, from phage display libraries using methods known to those of ordinary skill in the art. Furthermore, antibody reagents include single domain antibodies, such as camelid antibodies.

[00111] The V_H and V_L regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" ("FR"). The extent of the framework region and CDRs has been precisely defined (see, Kabat, E. A. et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia et al., J. Mol. Biol. 196:901-917, 1987; each of which is incorporated by reference herein in its entirety). Each V_H and V_L is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[00112] In one embodiment, the antibody or antibody reagent is not a human antibody or antibody reagent (i.e., the antibody or antibody reagent is mouse), but has been humanized. A "humanized antibody or antibody reagent" refers to a non-human antibody or antibody reagent that has been modified at the protein sequence level to increase its similarity to antibody or antibody reagent variants produced naturally in humans. One approach to humanizing antibodies employs the grafting of murine or other non-human CDRs onto human antibody frameworks.

[00113] In one embodiment, the extracellular target binding domain of a CAR comprises or consists essentially of a single-chain Fv (scFv) fragment created by fusing the V_H and V_L domains of an antibody, generally a monoclonal antibody, via a flexible linker peptide. In various embodiments, the scFv is fused to a transmembrane domain and to a T cell receptor intracellular signaling domain, e.g., an engineered intracellular signaling domain as described herein. In another embodiment, the extracellular target binding domain of a CAR comprises a camelid antibody.

[00114] Antibody binding domains and ways to select and clone them are well-known to those of ordinary skill in the art. In one embodiment, the antibody reagent is an anti-GARP antibody reagent and comprises the sequence of SEQ ID NO: 3 or 25, or comprises a sequence with at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to the sequence of SEQ ID NO: 3 or 25. In another embodiment, the antibody reagent is an anti-LAP antibody reagent and comprises the sequence of SEQ ID NO: 9 or 15, or comprises a sequence with at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to the sequence of SEQ ID

NO: 9 or 15. In another embodiment, the antibody reagent is an anti-EGFR or anti-EGFRvIII antibody reagent and comprises the sequence of SEQ ID NO: 21, 27, 33, 36, 42, 45, 55, 57, or 65, or comprises a sequence with at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to the sequence of SEQ ID NO: 21, 27, 33, 36, 42, 45, 55, 57, or 65. In another embodiment, the antibody reagent is an anti-CD19 antibody reagent and comprises the sequence of SEQ ID NO: 51 or 63, or comprises a sequence with at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to the sequence of SEQ ID NO: 51 or 63. In another embodiment, the antibody reagent is an anti-CD3 antibody reagent and comprises the sequence of SEQ ID NO: 34, 43, 52, 56, or 64, or comprises a sequence with at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to the sequence of SEQ ID NO: 34, 43, 52, 56, or 64. In various examples, the antibody can be selected from C225, 3C10, Cetuximab, and 2173.

[00115] In one embodiment, the CARs useful in the technology described herein comprise at least two antigen-specific targeting regions, an extracellular domain, a transmembrane domain, and an intracellular signaling domain. In such embodiments, the two or more antigen-specific targeting regions target at least two different antigens and may be arranged in tandem and separated by linker sequences. In another embodiment, the CAR is a bispecific CAR. A bispecific CAR is specific to two different antigens.

[00116] **Target/Antigen**

[00117] Any cell-surface moiety can be targeted by a CAR. Often, the target will be a cell-surface polypeptide that may be differentially or preferentially expressed on a cell that one wishes to target for a T cell response. To target Tregs, antibody reagents can be targeted against, e.g., Glycoprotein A Repetitions Predominant (GARP), latency-associated peptide (LAP), CD25, CTLA-4, ICOS, TNFR2, GITR, OX40, 4-1BB, and LAG-3. To target tumors or cancer cells, antibody domains can be targeted against, e.g., EGFR or EGFRvIII, as described herein. Targeting tumor antigens or tumor-associated antigens that are specific to the tumors can provide a means to target tumor cells while avoiding or at least limiting collateral damage to non-tumor cells or tissues. Non-limiting examples of additional tumor antigens, tumor-associated antigens, or other antigen of interest include CD19, CD37, BCMA (tumor necrosis factor receptor superfamily member 17 (TNFRSF17); NCBI Gene ID: 608; NCBI Ref Seq NP_001183.2) and mRNA (e.g., NCBI Ref Seq NM_ 001192.2), CEA, immature laminin receptor, TAG-72, HPV E6 and E7, BING-4, calcium-activated chloride channel 2, cyclin B1, 9D7, Ep-CAM, EphA3, her2/neu, telomerase, mesotheliun, SAP-1, survivin, BAGE family, CAGE family, GAGE family, MAGE family, SAGE family, XAGE family, NY-ESO-1/LAGE-1, PRAME, SSX-2, Melan-A/MART-1, gp100/pmel17, tyrosinase, TRP-1/-2, MC1R, BRCA1/2, CDK4, MART-2, p53, Ras, MUC1, TGF-βRII, IL-15, IL13Ra2, and CSF1R.

[00118] **Transmembrane Domain**

[00119] Each CAR as described herein includes a transmembrane domain that joins the extracellular target-binding domain to the intracellular signaling domain.

[00120] As used herein, "transmembrane domain" (TM domain) refers to the generally hydrophobic region of the CAR which crosses the plasma membrane of a cell. The TM domain can be the

transmembrane region or fragment thereof of a transmembrane protein (for example a Type I transmembrane protein or other transmembrane protein), an artificial hydrophobic sequence, or a combination thereof. While specific examples are provided herein and used in the Examples, other transmembrane domains will be apparent to those of skill in the art and can be used in connection with alternate embodiments of the technology. A selected transmembrane region or fragment thereof would preferably not interfere with the intended function of the CAR. As used in relation to a transmembrane domain of a protein or polypeptide, "fragment thereof" refers to a portion of a transmembrane domain that is sufficient to anchor or attach a protein to a cell surface.

[00121] In one embodiment, the transmembrane domain of a CAR or fragment thereof is derived from or comprises the transmembrane domain of CD8 (e.g., any one of SEQ ID NOs: 4, 10, 16, 22, 28, 37, 46, 58, or 66, or variants thereof). In an alternate embodiment, the transmembrane domain or fragment thereof of the CAR described herein comprises a transmembrane domain selected from the transmembrane domain of an alpha, beta or zeta chain of a T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, KIRDS2, OX40, CD2, CD27, LFA-1 (CDI la, CD18), ICOS (CD278), 4-1BB (CD137), 4-1BBL, GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRFI), CD160, CD19, IL2R beta, IL2R gamma, IL7R a, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CDI ld, ITGAE, CD103, ITGAL, CDI la, LFA-1, ITGAM, CDI lb, ITGAX, CDI lc, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1(CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRT AM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Lyl08), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKp44, NKp30, NKp46, NKG2D, and/or NKG2C.

[00122] CD8 is an antigen preferentially found on the cell surface of cytotoxic T lymphocytes. CD8 mediates cell-cell interactions within the immune system, and acts as a T cell co-receptor. CD8 consists of an alpha (CD8α) and beta (CD8β) chain. CD8a sequences are known for a number of species, e.g., human CD8a, (NCBI Gene ID: 925) polypeptide (e.g., NCBI Ref Seq NP_001139345.1) and mRNA (e.g., NCBI Ref Seq NM_ 000002.12). CD8 can refer to human CD8, including naturally occurring variants, molecules, and alleles thereof. In some embodiments of any of the aspects, e.g., in veterinary applications, CD8 can refer to the CD8 of, e.g., dog, cat, cow, horse, pig, and the like. Homologs and/or orthologs of human CD8 are readily identified for such species by one of skill in the art, e.g., using the NCBI ortholog search function or searching available sequence data for a given species for sequence similar to a reference CD8 sequence.

[00123] 4-1BBL is a type 2 transmembrane glycoprotein belonging to the TNF superfamily. 4-1BBL is expressed on activated T lymphocytes. 4-1BBL sequences are known for a number of species, e.g., human 4-1BBL, also known as TNFSF9 (NCBI Gene ID: 8744) polypeptide (e.g., NCBI Ref Seq NP_003802.1) and mRNA (e.g., NCBI Ref Seq NM_003811.3). 4-1BBL can refer to human 4-1BBL, including naturally occurring variants, molecules, and alleles thereof. In some embodiments of any of the aspects, e.g., in veterinary applications, 4-1BBL can refer to the 4-1BBL of, e.g., dog, cat, cow, horse, pig, and the like. Homologs and/or orthologs of human 4-1BBL are readily identified for such species by one of skill in the art, e.g., using the NCBI ortholog search function or searching available sequence data for a given species for sequence similar to a reference 4-1BBL sequence.

[00124] Co-stimulatory Domain

[00125] Each CAR described herein optionally comprises the intracellular domain of one or more co-stimulatory molecule or co-stimulatory domain. As used herein, the term "co-stimulatory domain" refers to an intracellular signaling domain of a co-stimulatory molecule. Co-stimulatory molecules are cell surface molecules other than antigen receptors or Fc receptors that provide a second signal required for efficient activation and function of T lymphocytes upon binding to antigen. In one example, a 4-1BB intracellular domain (ICD) can be used (see, e.g., below and SEQ ID NOs: 5, 11, 17, 23, 29, 38, 47, 59, 67, or variants thereof). Additional illustrative examples of such co-stimulatory molecules include CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD223 (LAG3), CD270 (HVEM), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), DAP10, LAT, NKD2C SLP76, TRIM, and ZAP70. In one embodiment, the intracellular domain is the intracellular domain of 4-1BB. 4-1BB (CD137; TNFRS9) is an activation-induced costimulatory molecule, and is an important regulator of immune responses.

[00126] Intracellular Signaling Domain

[00127] CARs as described herein comprise an intracellular signaling domain. An "intracellular signaling domain," refers to the part of a CAR polypeptide that participates in transducing the message of effective CAR binding to a target antigen into the interior of the immune effector cell to elicit effector cell function, e.g., activation, cytokine production, proliferation and cytotoxic activity, including the release of cytotoxic factors to the CAR-bound target cell, or other cellular responses elicited following antigen binding to the extracellular CAR domain. In various examples, the intracellular signaling domain is from CD3ζ (see, e.g., below and SEQ ID NOs: 6, 12, 18, 24, 30, 39, 48, 60, 68, or variants thereof). Additional non-limiting examples of ITAM-containing intracellular signaling domains that are of particular use in the technology include those derived from TCRζ, FcRγ, FcRβ, CD3γ, CD3θ, CD3δ, CD3ε, CD3ζ, CD22, CD79a, CD79b, and CD66d.

[00128] CD3 is a T cell co-receptor that facilitates T lymphocyte activation when simultaneously engaged with the appropriate co-stimulation (e.g., binding of a co-stimulatory molecule). A CD3 complex consists of 4 distinct chains; mammal CD3 consists of a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with a molecule known as the T cell receptor (TCR) and the CD3ζ to generate an activation signal in T lymphocytes. A complete TCR complex comprises a TCR, CD3ζ, and the complete CD3 complex.

[00129] In some embodiments of any aspect, a CAR polypeptide described herein comprises an intracellular signaling domain that comprises an Immunoreceptor Tyrosine-based Activation Motif or ITAM from CD3 zeta (CD3ζ). In some embodiments of any aspect, the ITAM comprises three motifs of ITAM of CD3ζ (ITAM3). In some embodiments of any aspect, the three motifs of ITAM of CD3ζ are not mutated and, therefore, include native or wild-type sequences. In some embodiments, the CD3ζ sequence comprises the sequence of a CD3ζ as set forth in the sequences provided herein.

[00130] Individual CAR and other construct components as described herein can be used with one another and swapped in and out of various constructs described herein, as can be determined by those of skill in the art. Each of these components can comprise or consist of any of the corresponding sequences set forth herein, or variants thereof.

[00131] A more detailed description of CARs and CAR T cells can be found in Maus et al., Blood 123:2624-2635, 2014; Reardon et al., Neuro-Oncology 16:1441-1458, 2014; Hoyos et al., Haematologica 97:1622, 2012; Byrd et al., J. Clin. Oncol. 32:3039-3047, 2014; Maher et al., Cancer Res 69:4559-4562, 2009; and Tamada et al., Clin. Cancer Res. 18:6436-6445, 2012; each of which is incorporated by reference herein in its entirety.

[00132] In one embodiment, the CAR further comprises a linker domain. As used herein "linker domain" refers to an oligo- or polypeptide region from about 2 to 100 amino acids in length, which links together any of the domains/regions of the CAR as described herein. In some embodiment, linkers can include or be composed of flexible residues such as glycine and serine so that the adjacent protein domains are free to move relative to one another. Longer linkers may be used when it is desirable to ensure that two adjacent domains do not sterically interfere with one another. Linkers may be cleavable or non-cleavable. Examples of cleavable linkers include 2A linkers (for example T2A), 2A-like linkers or functional equivalents thereof and combinations thereof. In various examples, linkers having sequences as set forth herein, or variants thereof, are used. It is to be understood that the indication of a particular linker in a construct in a particular location does not mean that only that linker can be used there. Rather, different linker sequences (e.g., P2A and T2A) can be swapped with one another (e.g., in the context of the constructs of the present invention), as can be determined by those of skill in the art. In one embodiment, the linker region is T2A derived from Thosea asigna virus. Non-limiting examples of linkers that can be used in this technology include T2A, P2A, E2A, BmCPV2A, and BmIFV2A. Linkers such as these can be used in the context of polyproteins, such as those described below. For example, they can be used to separate a CAR component of a polyprotein from a therapeutic agent (e.g., an antibody, such as a scFv, single domain antibody (e.g., a camelid antibody), or a bispecific antibody (e.g., a BiTE)) component of a polyprotein (see below).

[00133] In some embodiments, a CAR as described herein optionally further comprises a reporter molecule, e.g., to permit for non-invasive imaging (e.g., positron-emission tomography PET scan). In a bispecific CAR that includes a reporter molecule, the first extracellular binding domain and the second extracellular binding domain can include different or the same reporter molecule. In a bispecific CAR T cell, the first CAR and the second CAR can express different or the same reporter molecule. In another embodiment, a CAR as described herein further comprises a reporter molecule (for example hygromycin phosphotransferase (hph)) that can be imaged alone or in combination with a substrate or chemical (for example 9-[4-[^{18}F]fluoro-3-(hydroxymethyl)butyl]guanine ([^{18}F]FHBG)). In another embodiment, a CAR as described herein further comprises nanoparticles at can be readily imaged using non-invasive techniques (e.g., gold nanoparticles (GNP) functionalized with $^{64}Cu^{2+}$). Labeling of CAR T cells for non-invasive imaging is reviewed, for example in Bhatnagar et al., Integr. Biol. (Camb). 5(1):231-238, 2013, and Keu et al., Sci. Transl. Med. 18; 9(373), 2017, which are incorporated herein by reference in their entireties.

[00134] GFP and mCherry are demonstrated herein as fluorescent tags useful for imaging a CAR expressed on a T cell (e.g., a CAR T cell). It is expected that essentially any fluorescent protein known in the art can be used as a fluorescent tag for this purpose. For clinical applications, the CAR need not include a fluorescent tag or fluorescent protein. In each instance of particular constructs provided herein, therefore, any markers present in the constructs can be removed. The invention includes the constructs with or without the markers. Accordingly, when a specific construct is referenced herein, it can be

considered with or without any markers or tags (including, e.g., histidine tags) as being included within the invention.

[00135] In one embodiment, the CAR polypeptide sequence corresponds to, comprises, or comprises a sequence with at least 80%, at least 85%, 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 greater sequence identity of a sequence selected from SEQ ID NOs: 1, 7, or 13, or the combination of SEQ ID NOs: 21-24, 27-30, 36-39, 45-48, 57-60, or 65-68. As can be determined by those of skill in the art, various functionally similar or equivalent components of these CARs can be swapped or substituted with one another, as well as other similar or functionally equivalent components known in the art or listed herein.

[00136] **Antibodies and Related Molecules Delivered by CAR T Cells**

[00137] As noted above, the CAR T cells of the invention can optionally be used to deliver antibody reagents (or other therapeutic molecules, e.g., cytokines) to tumors, such as to the tumor microenvironment. In various embodiments, the antibody reagents are encoded by the same nucleic acid molecule as the CAR, thus facilitating transduction of cells (e.g., T cells) to express both a CAR and an antibody reagent. In such examples, the antibody reagent can be expressed, e.g., such that it is separated from the CAR (and optionally other proteins, e.g., markers) by cleavable linker sequences (e.g., a 2A linker, such as, e.g., P2A or T2A; see above). The antibody reagent can be expressed under the control of the same promoter as the CAR (e.g., by an EF1α promoter), and can be constitutively expressed. In other examples, the antibody reagent is expressed under the control of an inducible promoter, e.g., a promoter that is expressed upon T cell activation (e.g., an NFAT promoter). Such an inducible promoter can be used, e.g., to ensure that the antibody is expressed only upon T cell activation, and thus only, e.g., when the CAR T cell is within the tumor microenvironment, to which locale it may be advantageous to have antibody production limited. As is understood in the art, the CAR coding sequences can be 5' or 3' to the antibody reagent coding sequences in various vector designs within the invention.

[00138] In various examples, the antibodies expressed within a CAR T cell (e.g., from the same nucleic acid molecule as the CAR) are single chain antibodies or single domain antibodies as described herein (scFV or camelids). In the case of single chain antibodies, the chains may be in the order L-H or H-L, and optionally may be separated from one another by a linker (e.g., a glycine-based linker).

[00139] In various examples, the antibodies are bi-specific antibodies including, e.g., bispecific T cell engagers (BiTEs). Such molecules can target T cells (e.g., by binding CD3) as well as a tumor antigen (e.g., EGFR, EGFRvIII, or CD19; also see above), and can be used to augment the T cell response in, e.g., the tumor microenvironment. The two components of a BiTE can optionally be separated from one another by a linker (e.g., a glycine-based linker).

[00140] Antibody reagents can be targeted against, e.g., tumor antigens or Treg antigens, such as those described herein, or other antigens (e.g., EGFR, EGFRvIII, CD19, CTLA4, CD25, GARP, LAP, IL-15, IL13Ra2, CSF1R etc.)

[00141] In addition to optionally delivering antibody reagents, as described herein, the CAR T cells of the invention can be used to delivery other therapeutic molecules including, e.g., cytokines and toxins.

[00142] Other components of CARs and related constructs (or variants thereof), as described herein, such as Igκ (e.g., SEQ ID NO: 32, 41, 50, 54, 62, or variants thereof), CD8 leader (e.g., SEQ ID NO: 2,

14, 20, or variants thereof), and related sequences, can be selected for use in making constructs of the invention, as will be apparent to those of skill in the art.

[00143] Cells and Therapy

[00144] One aspect of the technology described herein relates to a mammalian cell comprising any of the CAR polypeptides described herein (optionally together with another therapeutic molecule, e.g., an antibody reagent (e.g., a scFv, a camelid antibody, or a BiTE) or a cytokine); or a nucleic acid encoding any of the CAR polypeptides described herein (optionally together with another therapeutic molecule, e.g., an antibody reagent (e.g., a scFv, a camelid antibody, or a cytokine). In one embodiment, the mammalian cell comprises an antibody, antibody reagent, antigen-binding portion thereof, any of the CARs described herein, or a cytokine, or a nucleic acid encoding such an antibody, antibody reagent, antigen-binding portion thereof, any of the CARs described herein, or a cytokine. The mammalian cell or tissue can be of human, primate, hamster, rabbit, rodent, cow, pig, sheep, horse, goat, dog or cat origin, but any other mammalian cell may be used. In a preferred embodiment of any aspect, the mammalian cell is human.

[00145] In one embodiment, the cell is a T cell. In alternate embodiments of any aspect, the cell is an immune cell. As used herein, "immune cell" refers to a cell that plays a role in the immune response. Immune cells are of hematopoietic origin, and include lymphocytes, such as B cells and T cells; natural killer cells; myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes. In some embodiments, the cell is a T cell; a NK cell; a NKT cell; lymphocytes, such as B cells and T cells; and myeloid cells, such as monocytes, macrophages, eosinophils, mast cells, basophils, and granulocytes.

[00146] In one embodiment, the cell is obtained from an individual having or diagnosed as having cancer, a plasma cell disorder, or autoimmune disease.

[00147] "Cancer" as used herein can refer to a hyperproliferation of cells whose unique trait, loss of normal cellular control, results in unregulated growth, lack of differentiation, local tissue invasion, and metastasis, and can be glioblastoma, glioma, leukemia, lymphoma, multiple myeloma, or a solid tumor. Non-limiting examples of leukemia include acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia (CLL). In one embodiment, the cancer is ALL or CLL. Non-limiting examples of lymphoma include diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell lymphoma (MCL), marginal zone lymphomas, Burkitt lymphoma, hairy cell leukemia (HCL), and T cell lymphoma (e.g., peripheral T cell lymphoma (PTCL), including cutaneous T cell lymphoma (CTCL) and anaplastic large cell lymphoma (ALCL)). In one embodiment, the cancer is DLBCL or follicular lymphoma. Non-limiting examples of solid tumors include adrenocortical tumor, alveolar soft part sarcoma, carcinoma, chondrosarcoma, colorectal carcinoma, desmoid tumors, desmoplastic small round cell tumor, endocrine tumors, endodermal sinus tumor, epithelioid hemangioendothelioma, Ewing sarcoma, germ cell tumors (solid tumor), giant cell tumor of bone and soft tissue, hepatoblastoma, hepatocellular carcinoma, melanoma, nephroma, neuroblastoma, non-rhabdomyosarcoma soft tissue sarcoma (NRSTS), osteosarcoma, paraspinal sarcoma, renal cell carcinoma, retinoblastoma, rhabdomyosarcoma, synovial sarcoma, and Wilms tumor. Solid tumors can be found in bones, muscles, or organs, and can be sarcomas or carcinomas. It is contemplated that any aspect of the technology

described herein can be used to treat all types of cancers, including cancers not listed in the instant application. As used herein, the term "tumor" refers to an abnormal growth of cells or tissues, e.g., of malignant type or benign type.

[00148] As used herein, an "autoimmune disease or disorder" is characterized by the inability of one's immune system to distinguish between a foreign cell and a healthy cell. This results in one's immune system targeting one's healthy cells for programmed cell death. Non-limiting examples of an autoimmune disease or disorder include inflammatory arthritis, type 1 diabetes mellitus, multiples sclerosis, psoriasis, inflammatory bowel diseases, SLE, and vasculitis, allergic inflammation, such as allergic asthma, atopic dermatitis, and contact hypersensitivity. Other examples of auto-immune-related disease or disorder, but should not be construed to be limited to, include rheumatoid arthritis, multiple sclerosis (MS), systemic lupus erythematosus, Graves' disease (overactive thyroid), Hashimoto's thyroiditis (underactive thyroid), celiac disease, Crohn's disease and ulcerative colitis, Guillain-Barre syndrome, primary biliary sclerosis/cirrhosis, sclerosing cholangitis, autoimmune hepatitis, Raynaud's phenomenon, scleroderma, Sjogren's syndrome, Goodpasture's syndrome, Wegener's granulomatosis, polymyalgia rheumatica, temporal arteritis/giant cell arteritis, chronic fatigue syndrome CFS), psoriasis, autoimmune Addison's Disease, ankylosing spondylitis, acute disseminated encephalomyelitis, antiphospholipid antibody syndrome, aplastic anemia, idiopathic thrombocytopenic purpura, myasthenia gravis, opsoclonus myoclonus syndrome, optic neuritis, Ord's thyroiditis, pemphigus, pernicious anaemia, polyarthritis in dogs, Reiter's syndrome, Takayasu's arteritis, warm autoimmune hemolytic anemia, Wegener's granulomatosis and fibromyalgia (FM).

[00149] In one embodiment, the mammalian cell is obtained for a patient having an immune system disorder that results in abnormally low activity of the immune system, or immune deficiency disorders, which hinders one's ability to fight a foreign agent (e.g., a virus or bacterial cell).

[00150] A plasma cell is a white blood cell produces from B lymphocytes which function to generate and release antibodies needed to fight infections. As used herein, a "plasma cell disorder or disease" is characterized by abnormal multiplication of a plasma cell. Abnormal plasma cells are capable of "crowding out" healthy plasma cells, which results in a decreased capacity to fight a foreign object, such as a virus or bacterial cell. Non-limiting examples of plasma cell disorders include amyloidosis, Waldenstrom's macroglobulinemia, osteosclerotic myeloma (POEMS syndrome), monoclonal gammopathy of unknown significance (MGUS), and plasma cell myeloma.

[00151] T cells can be obtained from a subject using standard techniques known in the field. For example, T cells can be isolated from peripheral blood taken from a donor or patient. T cells can be isolated from a mammal. Preferably, T cells are isolated from a human.

[00152] A cell, for example a T cell, can be engineered to comprise any of the CAR polypeptides described herein (including CAR polypeptides that are cleavably linked to antibody reagents or cytokines, as described herein); or a nucleic acid encoding any of the CAR polypeptides (and optionally also a genetically encoded antibody reagent or cytokine) described herein. In one embodiment, the any of the CAR polypeptides (optionally together with an antibody reagent as described herein or a cytokine) described herein are expressed from a lentiviral vector. The lentiviral vector is used to express the CAR polypeptide (and optionally also the antibody reagent or cytokine) in a cell using infection standard techniques.

[00153] Retroviruses, such as lentiviruses, provide a convenient platform for delivery of nucleic acid sequences encoding a gene or chimeric gene of interest. A selected nucleic acid sequence can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells, e.g., *in vitro* or *ex vivo*. Retroviral systems are well known in the art and are described in, for example, U.S. Patent No. 5,219,740; Kurth and Bannert (2010) "Retroviruses: Molecular Biology, Genomics and Pathogenesis" Calster Academic Press (ISBN:978-1-90455-55-4); and Hu et al., Pharmacological Reviews 52:493-512, 2000; which are each incorporated by reference herein in their entirety. Lentiviral system for efficient DNA delivery can be purchased from OriGene; Rockville, MD. In alternative embodiments, the CAR polypeptide (and optionally the antibody reagent or cytokine) of any of the CARS described herein are expressed in the mammalian cell via transfection or electroporation of an expression vector comprising nucleic acid encoding the CAR. Transfection or electroporation methods are known in the art.

[00154] Efficient expression of the CAR polypeptide (and optionally the antibody reagent or cytokine) of any of the polypeptides described herein can be assessed using standard assays that detect the mRNA, DNA, or gene product of the nucleic acid encoding the CAR (and optional antibody reagent or cytokine). For example, RT-PCR, FACS, northern blotting, western blotting, ELISA, or immunohistochemistry.

[00155] In one embodiment, the CAR polypeptide (and optional antibody reagent or cytokine) described herein is constitutively expressed. In one embodiment, the CAR polypeptide is constitutively expressed and the optional antibody reagent or cytokine is inducible expressed. In one embodiment, the CAR polypeptide (and optional antibody reagent or cytokine) described herein is encoded by recombinant nucleic acid sequence.

[00156] One aspect of the technology described herein relates to a method of treating cancer, a plasma cell disorder, or an autoimmune disease in a subject in need thereof, the method comprising: engineering a T cell to comprise any of the CAR polypeptides (and optional antibody reagents or cytokines) described herein on the T cell surface; and administering the engineered T cell to the subject. In the case of cancer, the method can be for treating diagnosed cancer, preventing recurrence of cancer, or for use in an adjuvant or neoadjuvant setting.

[00157] One aspect of the technology described herein relates to a method of treating cancer, a plasma cell disorder, or an autoimmune disease in a subject in need thereof, the method comprising: administering the cell of any of the mammalian cells comprising the any of the CAR polypeptides (and optional antibody reagents or cytokines) described herein.

[00158] Cluster differentiation (CD) molecules are cell surface markers present on leukocytes. As a leukocyte differentiates and matures its CD profile changes. In the case that a leukocytes turns into a cancer cell (i.e., a lymphoma), its CD profile is important in diagnosing the disease. The treatment and prognosis of certain types of cancers is reliant on determining the CD profile of the cancer cell. "CDX+", wherein "X" is a CD marker, indicates the CD marker is present in the cancer cell, while "CDX-" indicates the marker is not present. One skilled in the art will be capable of assessing the CD molecules present on a cancer cell using standard techniques, for example, using immunofluorescence to detect commercially available antibodies bound to the CD molecules.

[00159] In some embodiments of any of the aspect, the engineered CAR-T cell is stimulated and/or activated prior to administration to the subject.

[00160] **Administration**

[00161] In some embodiments, the methods described herein relate to treating a subject having or diagnosed as having cancer, a plasma cell disease or disorder, or an autoimmune disease or disorder with a mammalian cell comprising any of the CAR polypeptides (and optional antibody reagents or cytokines) described herein, or a nucleic acid encoding any of the CAR polypeptides (and optional antibody reagents or cytokines) described herein. As used herein, a "CAR T cells as described herein" refers to a mammalian cell comprising any of the CAR polypeptides (and optional antibody reagents or cytokines) described herein, or a nucleic acid encoding any of the CAR polypeptides (and optional antibody reagents or cytokines) described herein. As used herein, a "condition" refers to a cancer, a plasma cell disease or disorder, or an autoimmune disease or disorder. Subjects having a condition can be identified by a physician using current methods of diagnosing the condition. Symptoms and/or complications of the condition, which characterize these conditions and aid in diagnosis are well known in the art and include but are not limited to, fatigue, persistent infections, and persistent bleeding. Tests that may aid in a diagnosis of, e.g., the condition, but are not limited to, blood screening and bone marrow testing, and are known in the art for a given condition. A family history for a condition, or exposure to risk factors for a condition can also aid in determining if a subject is likely to have the condition or in making a diagnosis of the condition.

[00162] The compositions described herein can be administered to a subject having or diagnosed as having a condition. In some embodiments, the methods described herein comprise administering an effective amount of activated CAR T cells described herein to a subject in order to alleviate a symptom of the condition. As used herein, "alleviating a symptom of the condition" is ameliorating any condition or symptom associated with the condition. As compared with an equivalent untreated control, such reduction is by at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, 99% or more as measured by any standard technique. A variety of means for administering the compositions described herein to subjects are known to those of skill in the art. In one embodiment, the compositions described herein are administered systemically or locally. In a preferred embodiment, the compositions described herein are administered intravenously. In another embodiment, the compositions described herein are administered at the site of a tumor.

[00163] The term "effective amount" as used herein refers to the amount of activated CAR T cells needed to alleviate at least one or more symptom of the disease or disorder, and relates to a sufficient amount of the cell preparation or composition to provide the desired effect. The term "therapeutically effective amount" therefore refers to an amount of activated CAR T cells that is sufficient to provide a particular anti-condition effect when administered to a typical subject. An effective amount as used herein, in various contexts, would also include an amount sufficient to delay the development of a symptom of the disease, alter the course of a symptom disease (for example but not limited to, slowing the progression of a condition), or reverse a symptom of the condition. Thus, it is not generally practicable to specify an exact "effective amount." However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

[00164] Effective amounts, toxicity, and therapeutic efficacy can be evaluated by standard pharmaceutical procedures in cell cultures or experimental animals. The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of activated CAR T cells, which achieves a half-maximal inhibition of symptoms) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay, e.g., assay for bone marrow testing, among others. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

[00165] In one aspect of the technology, the technology described herein relates to a pharmaceutical composition comprising activated CAR T cells as described herein, and optionally a pharmaceutically acceptable carrier. The active ingredients of the pharmaceutical composition at a minimum comprise activated CAR T cells as described herein. In some embodiments, the active ingredients of the pharmaceutical composition consist essentially of activated CAR T cells as described herein. In some embodiments, the active ingredients of the pharmaceutical composition consist of activated CAR T cells as described herein. Pharmaceutically acceptable carriers for cell-based therapeutic formulation include saline and aqueous buffer solutions, Ringer's solution, and serum component, such as serum albumin, HDL and LDL. The terms such as “excipient,” “carrier,” “pharmaceutically acceptable carrier” or the like are used interchangeably herein.

[00166] In some embodiments, the pharmaceutical composition comprising activated CAR T cells as described herein can be a parenteral dose form. Since administration of parenteral dosage forms typically bypasses the patient's natural defenses against contaminants, the components apart from the CAR T cells themselves are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions. Any of these can be added to the activated CAR T cells preparation prior to administration.

[00167] Suitable vehicles that can be used to provide parenteral dosage forms of activated CAR T cells as disclosed within are well known to those skilled in the art. Examples include, without limitation: saline solution; glucose solution; aqueous vehicles including but not limited to, sodium chloride injection, Ringer's injection, dextrose injection, dextrose and sodium chloride injection, and lactated Ringer's injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and propylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate.

[00168] **Dosage**

[00169] “Unit dosage form” as the term is used herein refers to a dosage for suitable one administration. By way of example, a unit dosage form can be an amount of therapeutic disposed in a delivery device, e.g., a syringe or intravenous drip bag. In one embodiment, a unit dosage form is

administered in a single administration. In another, embodiment more than one unit dosage form can be administered simultaneously.

[00170] In some embodiments, the activated CAR T cells described herein are administered as a monotherapy, i.e., another treatment for the condition is not concurrently administered to the subject.

[00171] A pharmaceutical composition comprising the T cells described herein can generally 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. If necessary, T cell compositions can 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. Med. 319:1676, 1988).

[00172] In certain aspects, it may be desired to administer activated CAR T cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate T cells therefrom as described herein, and reinfuse the patient with these activated and expanded T cells. This process can be carried out multiple times every few weeks. In certain aspects, T cells can be activated from blood draws of from l0 cc to 400 cc. In certain aspects, T cells are activated from blood draws of 20 cc, 30 cc, 40 cc, 50 cc, 60 cc, 70 cc, 80 cc, 90 cc, or l00 cc.

[00173] Modes of administration can include, for example intravenous (i.v.) injection or infusion. The compositions described herein can be administered to a patient transarterially, intratumorally, intranodally, or intramedullary. In some embodiments, the compositions of T cells may be injected directly into a tumor, lymph node, or site of infection. In one embodiment, the compositions described herein are administered into a body cavity or body fluid (e.g., ascites, pleural fluid, peritoneal fluid, or cerebrospinal fluid).

[00174] In a particular exemplary aspect, subjects may undergo leukapheresis, wherein leukocytes are collected, enriched, or depleted *ex vivo* to select and/or isolate the cells of interest, e.g., T cells. These T cell isolates can be expanded by contact with an artificial APC, e.g., an aAPC expressing anti-CD28 and anti-CD3 CDRs, and treated such that one or more CAR constructs of the technology may be introduced, thereby creating a CAR T cell. Subjects in need thereof can subsequently undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. Following or concurrent with the transplant, subjects can receive an infusion of the expanded CAR T cells. In one embodiment, expanded cells are administered before or following surgery.

[00175] In some embodiments, lymphodepletion is performed on a subject prior to administering one or more CAR T cell as described herein. In such embodiments, the lymphodepletion can comprise administering one or more of melphalan, cytoxan, cyclophosphamide, and fludarabine.

[00176] The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices.

[00177] In some embodiments, a single treatment regimen is required. In others, administration of one or more subsequent doses or treatment regimens can be performed. For example, after treatment biweekly for three months, treatment can be repeated once per month, for six months or a year or longer. In some embodiments, no additional treatments are administered following the initial treatment.

[00178] The dosage of a composition as described herein can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. With respect to duration and frequency of treatment, it is typical for skilled clinicians to monitor subjects in order to determine when the treatment is providing therapeutic benefit, and to determine whether to administer further cells, discontinue treatment, resume treatment, or make other alterations to the treatment regimen. The dosage should not be so large as to cause adverse side effects, such as cytokine release syndrome. Generally, the dosage will vary with the age, condition, and sex of the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

[00179] **Combination therapy**

[00180] The activated CAR T cells described herein can optionally be used in combination with each other and with other known agents and therapies, as can determined to be appropriate by those of skill in the art. In one example, two or more CAR T cells targeting different Treg markers (e.g., GARP, LAP, etc.) can be administered in combination. In another example, two or more CAR T cells targeting different cancer antigens are administered in combination. In a further example, one or more CAR T cell targeting a Treg marker (e.g., GARP, LAP, etc.) and one or more CAR T cell targeting one or more tumor antigens are administered in combination.

[00181] Administered "in combination," as used herein, means that two (or more) different treatments are delivered to the subject during the course of the subject's affliction with the disorder, e.g., the two or more treatments are delivered after the subject has been diagnosed with the disorder and before the disorder has been cured or eliminated or treatment has ceased for other reasons. In some embodiments, the delivery of one treatment is still occurring when the delivery of the second begins, so that there is overlap in terms of administration. This is sometimes referred to herein as "simultaneous" or "concurrent delivery." In other embodiments, the delivery of one treatment ends before the delivery of the other treatment begins. In some embodiments of either case, the treatment is more effective because of combined administration. For example, the second treatment is more effective, e.g., an equivalent effect is seen with less of the second treatment, or the second treatment reduces symptoms to a greater extent, than would be seen if the second treatment were administered in the absence of the first treatment, or the analogous situation is seen with the first treatment. In some embodiments, delivery is such that the reduction in a symptom, or other parameter related to the disorder is greater than what would be observed with one treatment delivered in the absence of the other. The effect of the two treatments can be partially additive, wholly additive, or greater than additive. The delivery can be such that an effect of the first treatment delivered is still detectable when the second is delivered. The activated CAR T cells described herein and the at least one additional therapeutic agent can be administered simultaneously, in the same or in separate compositions, or sequentially. For sequential administration, the CAR-expressing cell described herein can be administered first, and the additional agent can be administered second, or the order of administration can be reversed. The CAR T therapy and/or other therapeutic agents, procedures or modalities can be administered during periods of active disorder, or during a period of remission or less active disease. The CAR T therapy can be administered before another treatment, concurrently with the treatment, post-treatment, or during remission of the disorder.

[00182] When administered in combination, the activated CAR T cells and the additional agent (e.g., second or third agent), or all, can be administered in an amount or dose that is higher, lower or the same

as the amount or dosage of each agent used individually, e.g., as a monotherapy. In certain embodiments, the administered amount or dosage of the activated CAR T cells, the additional agent (e.g., second or third agent), or all, is lower (e.g., at least 20%, at least 30%, at least 40%, or at least 50%) than the amount or dosage of each agent used individually. In other embodiments, the amount or dosage of the activated CAR T cells, the additional agent (e.g., second or third agent), or all, that results in a desired effect (e.g., treatment of cancer) is lower (e.g., at least 20%, at least 30%, at least 40%, or at least 50% lower) than the amount or dosage of each agent individually required to achieve the same therapeutic effect. In further embodiments, the activated CAR T cells described herein can be used in a treatment regimen in combination with surgery, chemotherapy, radiation, an mTOR pathway inhibitor, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludarabine, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, or a peptide vaccine, such as that described in Izumoto et al., J. Neurosurg. 108:963- 971, 2008.

[00183] In one embodiment, the activated CAR T cells described herein can be used in combination with a checkpoint inhibitor. Exemplary checkpoint inhibitors include anti-PD-1 inhibitors (Nivolumab, MK-3475, Pembrolizumab, Pidilizumab, AMP-224, AMP-514), anti-CTLA4 inhibitors (Ipilimumab and Tremelimumab), anti-PDL1 inhibitors (Atezolizumab, Avelomab, MSB0010718C, MEDI4736, and MPDL3280A), and anti-TIM3 inhibitors.

[00184] In one embodiment, the activated CAR T cells described herein can be used in combination with a chemotherapeutic agent. Exemplary chemotherapeutic agents include an anthracycline (e.g., doxorubicin (e.g., liposomal doxorubicin)), a vinca alkaloid (e.g., vinblastine, vincristine, vindesine, vinorelbine), an alkylating agent (e.g., cyclophosphamide, decarbazine, melphalan, ifosfamide, temozolomide), an immune cell antibody (e.g., alemtuzamab, gemtuzumab, rituximab, tositumomab), an antimetabolite (including, e.g., folic acid antagonists, pyrimidine analogs, purine analogs and adenosine deaminase inhibitors (e.g., fludarabine)), an mTOR inhibitor, a TNFR glucocorticoid induced TNFR related protein (GITR) agonist, a proteasome inhibitor (e.g., aclacinomycin A, gliotoxin or bortezomib), an immunomodulator such as thalidomide or a thalidomide derivative (e.g., lenalidomide). General chemotherapeutic agents considered for use in combination therapies include anastrozole (Arimidex®), bicalutamide (Casodex®), bleomycin sulfate (Blenoxane®), busulfan (Myleran®), busulfan injection (Busulfex®), capecitabine (Xeloda®), N4-pentoxycarbonyl-5- deoxy-5-fluorocytidine, carboplatin (Paraplatin®), carmustine (BiCNU®), chlorambucil (Leukeran®), cisplatin (Platinol®), cladribine (Leustatin®), cyclophosphamide (Cytoxan® or Neosar®), cytarabine, cytosine arabinoside (Cytosar-U®), cytarabine liposome injection (DepoCyt®), dacarbazine (DTIC-Dome®), dactinomycin (Actinomycin D, Cosmegan), daunorubicin hydrochloride (Cerubidine®), daunorubicin citrate liposome injection (DaunoXome®), dexamethasone, docetaxel (Taxotere®), doxorubicin hydrochloride (Adriamycin®, Rubex®), etoposide (Vepesid®), fludarabine phosphate (Fludara®), 5- fluorouracil (Adrucil®, Efudex®), flutamide (Eulexin®), tezacitibine, Gemcitabine (difluorodeoxycitidine), hydroxyurea (Hydrea®), Idarubicin (Idamycin®), ifosfamide (IFEX®), irinotecan (Camptosar®), L-asparaginase (ELSPAR®), leucovorin calcium, melphalan (Alkeran®), 6-mercaptopurine (Purinethol®), methotrexate (Folex®), mitoxantrone (Novantrone®), mylotarg, paclitaxel (Taxol®), phoenix (Yttrium90/MX-DTPA), pentostatin, polifeprosan 20 with carmustine implant (Gliadel®), tamoxifen citrate (Nolvadex®), teniposide (Vumon®), 6-thioguanine,

thiotepa, tirapazamine (Tirazone®), topotecan hydrochloride for injection (Hycamptin®), vinblastine (Velban®), vincristine (Oncovin®), and vinorelbine (Navelbine®). Exemplary alkylating agents include, without limitation, nitrogen mustards, ethylenimine derivatives, alkyl sulfonates, nitrosoureas and triazenes): uracil mustard (Aminouracil Mustard®, Chlorethaminacil®, Demethyldopan®, Desmethyldopan®, Haemanthamine®, Nordopan®, Uracil nitrogen mustard®, Uracillost®, Uracilmostaza®, Uramustin®, Uramustine®), chlormethine (Mustargen®), cyclophosphamide (Cytoxan®, Neosar®, Clafen®, Endoxan®, Procytox®, Revimmune™), ifosfamide (Mitoxana®), melphalan (Alkeran®), Chlorambucil (Leukeran®), pipobroman (Amedel®, Vercyte®), triethylenemelamine (Hemel®, Hexalen®, Hexastat®), triethylenethiophosphoramine, Temozolomide (Temodar®), thiotepa (Thioplex®), busulfan (Busilvex®, Myleran®), carmustine (BiCNU®), lomustine (CeeNU®), streptozocin (Zanosar®), and Dacarbazine (DTIC-Dome®). Additional exemplary alkylating agents include, without limitation, Oxaliplatin (Eloxatin®); Temozolomide (Temodar® and Temodal®); Dactinomycin (also known as actinomycin-D, Cosmegen®); Melphalan (also known as L-PAM, L-sarcolysin, and phenylalanine mustard, Alkeran®); Altretamine (also known as hexamethylmelamine (HMM), Hexalen®); Carmustine (BiCNU®); Bendamustine (Treanda®); Busulfan (Busulfex® and Myleran®); Carboplatin (Paraplatin®); Lomustine (also known as CCNU, CeeNU®); Cisplatin (also known as CDDP, Platinol® and Platinol®-AQ); Chlorambucil (Leukeran®); Cyclophosphamide (Cytoxan® and Neosar®); Dacarbazine (also known as DTIC, DIC and imidazole carboxamide, DTIC-Dome®); Altretamine (also known as hexamethylmelamine (HMM), Hexalen®); Ifosfamide (Ifex®); Prednumustine; Procarbazine (Matulane®); Mechlorethamine (also known as nitrogen mustard, mustine and mechloroethamine hydrochloride, Mustargen®); Streptozocin (Zanosar®); Thiotepa (also known as thiophosphoamide, TESPA and TSPA, Thioplex®); Cyclophosphamide (Endoxan®, Cytoxan®, Neosar®, Procytox®, Revimmune®); and Bendamustine HC1 (Treanda®). Exemplary mTOR inhibitors include, e.g., temsirolimus; ridaforolimus (formally known as deferolimus, (IR,2R,45)-4-[(2R)-2 [(1R,95,125,15R,16E,18R,19R,21R,235,24E,26E,28Z,305,325,35R)-l,18-dihydroxy-19,30-dimethoxy-15,17,21,23, 29,35- hexamethyl-2,3,10,14,20-pentaoxo-l l,36-dioxa-4-azatricyclo[30.3.1.04'9] hexatriaconta- 16,24,26,28-tetraen-12-yl]propyl]-2-methoxycyclohexyl dimethylphosphinate, also known as AP23573 and MK8669, and described in PCT Publication No. WO 03/064383); everolimus (Afinitor® or RADOOl); rapamycin (AY22989, Sirolimus®); simapimod (CAS 164301-51-3); emsirolimus, (5-{2,4-Bis[(35,)-3-methylmorpholin-4-yl]pyrido[2,3-(i]pyrimidin-7-yl}-2- methoxyphenyl)methanol (AZD8055); 2-Amino-8-[iraw5,-4-(2-hydroxyethoxy)cyclohexyl]-6- (6-methoxy-3-pyridinyl)-4-methyl-pyrido[2,3-JJpyrimidin-7(8H)-one (PF04691502, CAS 1013101-36-4); and N2-[l,4-dioxo-4-[[4-(4-oxo-8-phenyl-4H-l-benzopyran-2- yl)morpholinium-4-yl]methoxy]butyl]-L-arginylglycyl-L-a-aspartylL-serine-, inner salt (SF1126, CAS 936487-67-1), and XL765. Exemplary immunomodulators include, e.g., afutuzumab (available from Roche®); pegfilgrastim (Neulasta®); lenalidomide (CC-5013, Revlimid®); thalidomide (Thalomid®), actimid (CC4047); and IRX-2 (mixture of human cytokines including interleukin 1, interleukin 2, and interferon γ, CAS 951209-71-5, available from IRX Therapeutics). Exemplary anthracyclines include, e.g., doxorubicin (Adriamycin® and Rubex®); bleomycin (lenoxane®); daunorubicin (dauorubicin hydrochloride, daunomycin, and rubidomycin hydrochloride, Cerubidine®); daunorubicin liposomal (daunorubicin citrate liposome, DaunoXome®); mitoxantrone (DHAD, Novantrone®); epirubicin (Ellence™); idarubicin (Idamycin®, Idamycin PFS®); mitomycin C (Mutamycin®); geldanamycin;

herbimycin; ravidomycin; and desacetylravidomycin. Exemplary vinca alkaloids include, e.g., vinorelbine tartrate (Navelbine®), Vincristine (Oncovin®), and Vindesine (Eldisine®)); vinblastine (also known as vinblastine sulfate, vincaleukoblastine and VLB, Alkaban-AQ® and Velban®); and vinorelbine (Navelbine®). Exemplary proteosome inhibitors include bortezomib (Velcade®); carfilzomib (PX- 171-007, (5)-4-Methyl-N-((5)-l-(((5)-4-methyl-l-((R)-2-methyloxiran-2-yl)-l-oxopentan-2- yl)amino)-l-oxo-3-phenylpropan-2-yl)-2-((5,)-2-(2-morpholinoacetamido)-4- phenylbutanamido)-pentanamide); marizomib (NPT0052); ixazomib citrate (MLN-9708); delanzomib (CEP-18770); and O-Methyl-N-[(2-methyl-5-thiazolyl)carbonyl]-L-seryl-O- methyl-N-[(lS')-2-[(2R)-2-methyl-2-oxiranyl]-2-oxo-l-(phenylmethyl)ethyl]- L-serinamide (ONX-0912).

[00185] One of skill in the art can readily identify a chemotherapeutic agent of use (e.g., see Physicians' Cancer Chemotherapy Drug Manual 2014, Edward Chu, Vincent T. DeVita Jr., Jones & Bartlett Learning; Principles of Cancer Therapy, Chapter 85 in Harrison's Principles of Internal Medicine, 18th edition; Therapeutic Targeting of Cancer Cells: Era of Molecularly Targeted Agents and Cancer Pharmacology, Chapters 28-29 in Abeloff's Clinical Oncology, 2013 Elsevier; and Fischer D. S. (ed.): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 2003).

[00186] In an embodiment, activated CAR T cells described herein are administered to a subject in combination with a molecule that decreases the level and/or activity of a molecule targeting GITR and/or modulating GITR functions, a molecule that decreases the Treg cell population, an mTOR inhibitor, a GITR agonist, a kinase inhibitor, a non-receptor tyrosine kinase inhibitor, a CDK4 inhibitor, and/or a BTK inhibitor.

[00187] **Efficacy**

[00188] The efficacy of activated CAR T cells in, e.g., the treatment of a condition described herein, or to induce a response as described herein (e.g., a reduction in cancer cells) can be determined by the skilled clinician. However, a treatment is considered "effective treatment," as the term is used herein, if one or more of the signs or symptoms of a condition described herein is altered in a beneficial manner, other clinically accepted symptoms are improved, or even ameliorated, or a desired response is induced, e.g., by at least 10% following treatment according to the methods described herein. Efficacy can be assessed, for example, by measuring a marker, indicator, symptom, and/or the incidence of a condition treated according to the methods described herein or any other measurable parameter appropriate. Treatment according to the methods described herein can reduce levels of a marker or symptom of a condition, e.g. by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80 % or at least 90% or more.

[00189] Efficacy can also be measured by a failure of an individual to worsen as assessed by hospitalization, or need for medical interventions (i.e., progression of the disease is halted). Methods of measuring these indicators are known to those of skill in the art and/or are described herein.

[00190] Treatment includes any treatment of a disease in an individual or an animal (some non-limiting examples include a human or an animal) and includes: (1) inhibiting the disease, e.g., preventing a worsening of symptoms (e.g., pain or inflammation); or (2) relieving the severity of the disease, e.g., causing regression of symptoms. An effective amount for the treatment of a disease means that amount which, when administered to a subject in need thereof, is sufficient to result in effective treatment as that term is defined herein, for that disease. Efficacy of an agent can be determined by assessing physical

indicators of a condition or desired response. It is well within the ability of one skilled in the art to monitor efficacy of administration and/or treatment by measuring any one of such parameters, or any combination of parameters. Efficacy of a given approach can be assessed in animal models of a condition described herein. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change in a marker is observed.

[00191] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior technology or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00192] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. Moreover, due to biological functional equivalency considerations, some changes can be made in protein structure without affecting the biological or chemical action in kind or amount. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00193] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[00194] The technology described herein is further illustrated by the following examples, which in no way should be construed as being further limiting.

[00195] EXAMPLES

[00196] Example 1. EGFRvIII-targeted CAR T cells

[00197] CAR T cells having an EGFRvIII antigen-binding moiety (e.g., CART-EGFRvIII cells) represent a promising cellular therapy for specific targeting of cytolytic cells to the tumor microenvironment, in part because EGFRvIII is specifically expressed on tumor tissue while generally

absent from healthy tissue. In this example, CART-EGFRvIII cells were tested in vitro and in vivo in two animal models.

[00198] T cells from leukapheresis products obtained from deidentified healthy donors were stimulated with Dynabeads (Human T-Activator CD3/CD28) at a bead to cell ratio of 3:1 and cultured in complete RPMI 1640 medium. Ten days following stimulation and lentivirus transduction, cells were frozen and stored for use in functional assays.

[00199] Initial tests were performed in vitro to characterize the ability of CAR-EGFRvIII cells to preferentially kill tumor cells relative to untransduced control cells in a twenty-hour luciferase-based assay, shown in Fig. 1. U87vIII, a human glioma cell line, was used as target cells. In vitro characterization demonstrates that EGFRvIII CAR T cells mediate significant and specific cytotoxicity against U87vIII cells (Fig. 1).

[00200] For in vivo experiments, U87vIII tumor cells were collected in logarithmic growth phase, washed, and administered to mice subcutaneously in a xenograft model of human glioblastoma (Figs. 2A and 2B) or intracranially in a model of human glioma (Figs. 3A and 3B). For intracranial administrations, the needle of a 50 microliter Hamilton syringe was positioned using a stereotactic frame at 2 mm to the right of the bregma and 4 mm below the surface of the skull at the coronal suture. For treatment, mice were infused once with CAR T cells (1 x 10^6 CAR-transduced T cells per mouse) via tail vein.

[00201] The potent antitumor effect observed in vitro was mirrored in the in vivo subcutaneous xenograft model of human glioblastoma (Figs. 2A and 2B). In this model, established, bulky tumors (top rows) responded to CART-EGFRvIII (Fig. 2B), whereas untransduced cells did not prevent tumor growth (Fig. 2A). In the murine model of intracranial human glioma, EGFRvIII CAR T cells slowed the growth of tumors and led to prolonged survival (Fig. 3B) relative to untransduced cells (Fig. 3A). Although tumor growth was abrogated, the effects were not as pronounced as those observed against subcutaneous tumors.

[00202] The presence of regulatory T cells (Tregs) was observed in human patient tumor tissues after treatment with CART-EGFRvIII cells (Figs. 4A and 4B). To determine if brain-infiltrating Tregs have a functional role in suppressing CART-EGFRvIII cells, an in vitro Treg suppression assay was performed in which CART-EGFRvIII cells (red) and glioma cells (green) were incubated in the presence of Tregs for 18 hours. Results were obtained by IncyCyte live cell analysis, as shown in Figs. 5A-5C. While non-specific CAR cells permitted proliferation of glioma cells (Figs. 5A and 5D, top line), CART-EGFRvIII cells killed glioma cells (Figs. 5B and 5D, bottom line). However, addition of Tregs in the co-culture significantly reduced the ability of CART-EGFRvIII cells to kill target glioma cells (Figs. 5C and 5D, middle line).

[00203] Example 2. Design and characterization of CAR T cells targeted to Treg-associated antigens

[00204] Figs. 6A-6C, 7A, and 7B show results of an experiment in which LAP and GARP were identified as Treg-associated markers on human peripheral blood cells. In particular, among human Tregs that were not activated ex vivo, approximately 27% expressed LAP, approximately 4% were double positive for LAP and GARP (Fig. 6B). Once activated ex vivo using anti-CD3, anti-CD8, and IL-2, approximately 30% expressed LAP, and the number of LAP/GARP double positive Tregs increased to 12.3% (Fig. 6C).

[00205] Next, CAR constructs encoding CARs targeting LAP and GARP were designed. Schematic illustrations of these constructs are shown in Figs. 8A-8D. Treg-targeting constructs include two LAP-targeting CARs (CAR-LAP-L-H (Fig. 8A) and CAR-LAP-H-L (Fig. 8B); in which each anti-LAP scFv contains a reversal in heavy (H) and light (L) chain arrangement), a GARP-targeting CAR construct (CAR-GARP; Fig. 8C), and an EGFR-targeting CAR construct further encoding an anti-GARP camelid antibody (CAR-EGFR-GARP; Fig. 8D). Transduction efficiencies of each construct were assessed using flow cytometry by measuring the percentage of mCherry-positive cells and are provided below.

[00206] Transduction efficiencies of Treg-targeted CAR constructs

CAR construct	ND47	ND48	ND50
pMGH 97 CAR-GARP (SEQ ID NO: 1)	68.0%	81.0%	72.8%
pMGH 99 CAR-LAP-H-L (SEQ ID NO: 7)	57.1%	79.5%	80.4%
pMGH 100 CAR-LAP-L-H (SEQ ID NO: 13)	72.2%	88.2%	90.1%
pMGH 105 CAR-EGFR-GARP (SEQ ID NO: 19)	N/A	N/A	51.2%

[00207] To test anti-LAP CART cells, CAR T cells were co-cultured with isolated Tregs expanded from the same donor and transduced to express GFP as a Treg marker. Tregs were activated overnight with anti-CD3 and anti-CD28 (Fig. 9B) or rested overnight (Fig. 9A) prior to the killing assay. 62,500 Tregs per well were plated. CARs were added at the indicated ratio to Tregs. Cultures were incubated for three days in the presence of 300 U/mL IL-2. Flow cytometry was performed on day 3 by collecting 30,000 events per well. Percent cytotoxicity was calculated as the percent reduction in GFP-positive cells compared to the untransduced T cell culture with Tregs. CART-LAP-H-L was more effective at killing non-activated Tregs in comparison to CART-LAP-L-H. LAP-targeted CAR T cells were then compared to GARP-targeted CART cells in an analogous Treg killing assay across two different donors at a CAR T cell-to-Treg ratio of 1:1 for four days (Figs. 10A and 10B). Figs. 11A and 11B characterize non-activated and activated Treg killing by LAP-targeted CAR T cells, relative to untransduced controls, by the number of target Tregs remaining at the end of a three-day coculture as a function of CAR T cell-to-Treg cell ratio. Figs. 11C and 11D show analogous data from the same donor, in which cytotoxicity is measured by luciferase expression.

[00208] To further characterize the effect of antigen expression on function of LAP- and GARP-targeted CAR T cells, immortalized cell lines were screened for LAP and GARP antigen-expression, and the cytotoxic effect by each CAR T cell was assessed. First, HUT78 cells, a cutaneous human CD4 T cell lymphocyte-derived cell line that expresses IL-2, was stained for GARP and LAP (Figs. 12A and 12B, respectively), and LAP expression by HUT78 cells was confirmed. Next, CART-LAP-H-L and CART-LAP-L-H cell-mediated cytotoxicity toward HUT78 cells by cytotoxicity assays (Figs. 13A and 13B). Next, SeAx, an IL-2 dependent human Sezary syndrome-derived cell, was stained for GARP and LAP (Figs. 14A and 14B, respectively), and expression of both antigens was confirmed. SeAx cells were cocultured

with CART-GARP cells, CART-LAP-H-L cells, CART-LAP-L-L cells, and untransduced cells to quantify CAR T cell-mediated killing at 24 hours (Fig. 15A) and 48 hours (Fig. 15B). Each CAR T exhibited superior SeAx target cell killing at 24 hours, with a more pronounced effect at 48 hours. CART-GARP and CART-LAP-H-L killed target SeAx cells with greater efficiency than CART-LAP-L-H cells by 48 hours.

[00209] Next, secretion of anti-GARP camelid antibodies by CART-EGFR-GARP cells was characterized by western blot (Figs. 16A-16C). Supernatant was collected from cultures containing CART-EGFR-GARP cells, treated in reducing and non-reducing conditions, and presence of a band between 10 and 15 kD was observed in the lane containing the non-reduced sample (Fig. 16C), confirming the presence of a camelid antibody.

[00210] **Example 3. Design and characterization of BiTE-secreting CAR T cells**

[00211] Another mechanism provided herein to enhance efficacy of CAR T cell activity within tumor microenvironments (e.g., to overcome immune regulation by Tregs) is through a CAR T cell that secretes a immune-modulating antibodies, such as a BiTE. Without wishing to be bound by theory, the present inventors have discovered that expression of an immune-modulating antibody (e.g., a BiTE) from a construct that also encodes a CAR can further amplify antitumor effects.

[00212] One exemplary nucleic acid construct, CAR-EGFR-BiTE-(EGFR-CD3), shown schematically in Fig. 17, includes a CAR-encoding polynucleotide operatively linked 5' to a BiTE-encoding polynucleotide. The CAR features a tumor-antigen binding domain that binds to EGFRvIII, which directs the CAR T cell to the microenvironment of an EGFRvIII-positive tumor. The BiTE binds at one domain to EGFR and at the other domain to CD3, as shown in Fig. 18, which can (a) further enhance binding avidity of the host CAR T cell to the tumor cell or (b) arm neighboring (e.g., endogenous) T cells against the tumor. The BiTE is flanked by cleavable linkers P2A and T2A to enable separate secretion of the BiTE, while the CAR is targeted to the cell surface. Other exemplary BiTE-encoding CAR constructs (e.g., encoding a BiTE targeting CD19) are depicted in Figs. 26A and 26B.

[00213] BiTE secretion by CART-EGFR-BiTE-(EGFR-CD3) cells was confirmed by isolating supernatant from cultures containing SupT1 cells transduced with CAR-EGFR-BiTE-(EGFR-CD3), calculating the concentration of BiTE in the supernatant based on OD450, and performing western blot analysis. The concentration of BiTE in the supernatant was 0.604 ng/mL. Results of a western blot experiment are shown in Fig. 19. A band in lane two at about 50-60 kD was observed, indicating the presence of BiTE molecules in the supernatant.

[00214] Next, binding of BiTE molecules was assessed by flow cytometry. HEK293T cells were transduced with CAR-EGFR-BiTE-(EGFR-CD3), and supernatants containing secreted BiTEs were collected and incubated with K562 cells (Fig. 20A) and Jurkat cells (Fig. 20B). As shown in Fig. 20A, BiTEs bound K562 cells expressing EGFR and did not bind K562 cells expressing CD19, confirming function of the EGFR-binding domain of the BiTE. As shown in Fig. 20B, CD3-expressing Jurkat cells showed stronger staining for BiTE after incubation with supernatant from CAR-EGFR-BiTE-(EGFR-CD3)-expressing HEK293T cells, compared to staining for BiTE after incubation with supernatant from untransduced HEK293T cells, indicating that BiTEs also functionally bind to CD3.

[00215] A similar experiment was conducted using SupT1 cells as transduction hosts for CAR-EGFR-BiTE-(EGFR-CD3). FIG. 21A shows BiTEs bound K562 cells expressing EGFR and did not bind K562 cells expressing CD19, confirming function of the EGFR-binding domain of the BiTE expressed by

transduced SupT1 cells. To confirm that BiTEs bound to CD3 expressed on the surface of the host SupT1 cell, the transduced SupT1 cells were stained for BiTE. Results, shown in Fig. 21B, confirm that transduced SupT1 cells stain positive for BiTEs. ND4 cells were also assessed for ability to secrete functional BiTEs upon transduction with CAR-EGFR-BiTE-(EGFR-CD3). FIG. 22A shows BiTEs secreted by transduced ND4 cells bound K562 cells expressing EGFR and did not bind K562 cells expressing CD19. As shown in Fig. 22B, BiTEs bound to CD3 expressed on the transduced ND4 cells from which they were secreted.

[00216] Next, the ability of BiTEs secreted from transduced CAR T cells was characterized in vitro. Supernatants containing BiTEs secreted from HEK293T cells transduced with CAR-EGFR-BiTE-(EGFR-CD3) were incubated with a coculture of untransduced ND4 cells and U87vIII target cells at varying ratios. As shown in Fig. 23, ND4 cells, when incubated with BiTE, in a dose-dependent manner, indicating that BiTEs were binding to both ND4-expressed CD3 and U87vIII-expressed EGFR to a degree sufficient to induce killing by ND4 cells.

[00217] To enable inducible expression of BiTE upon T cell activation, a construct containing an NFAT promoter was designed and synthesized. As shown in Fig. 24, the NFAT promoter precedes a GFP-encoding polynucleotide, and the construct further includes a downstream CAR-encoding polynucleotide driven by EF1α, a constitutive promoter. To confirm the inducible expression of GFP, GFP expression was assessed by FACS in response to TCR stimulation by PMA/ionomycin. As shown in Figs. 25A and 25B, stimulation triggered the expression of GFP. This inducible expression was inhibited by incubation with PEPvIII. Inducible BiTE constructs encoding CARs are designed by positioning the BiTE downstream of an inducible promoter, such as an NFAT promoter, as shown in Figs. 27A and 27B.

[00218] **Example 4. Sequence Information**

[00219] **Anti-GARP CAR - pMGH 97: CD8 Leader – anti-GARP – CD8 hinge + TM – 4-1BB – CD3z** (SEQ ID NO: 1) comprising CD8 leader sequence (amino acids 1-21 of SEQ ID NO: 1; SEQ ID NO: 2); anti-GARP camelid (amino acids 22-128 of SEQ ID NO: 1; SEQ ID NO: 3); CD8 hinge/TM domain (amino acids 129-197 of SEQ ID NO: 1; SEQ ID NO: 4); 4-1BB ICD (amino acids 198-239 of SEQ ID NO: 1; SEQ ID NO: 5); and CD3ζ (amino acids 240-351 of SEQ ID NO: 1; SEQ ID NO: 6).

MALPVTALLLPLALLLHAARPDIQMTQSPSSLSASLGDRVTITCQASQSISSYLAWYQQK
PGQAPNILIYGASRLKTGVPSRFSGSGSGTSFTLTISGLEAEDAGTYYCQQYASVPVTFG
QGTKVELKTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPL
AGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE
GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
(SEQ ID NO: 1)

[00220] CD8 leader sequence (amino acids 1-21 of SEQ ID NO:1; SEQ ID NO: 2)

MALPVTALLLPLALLLHAARP

[00221] anti-GARP camelid (amino acids 22-128 of SEQ ID NO:1; SEQ ID NO: 3)

DIQMTQSPSSLSASLGDRVTITCQASQSISSYLAWYQQKPGQAPNILIYGASRLK
TGVPSRFSGSGSGTSFTLTISGLEAEDAGTYYCQQYASVPVTFGQGTKVELK

[00222] CD8 hinge/TM domain (amino acids 129-197 of SEQ ID NO:1; SEQ ID NO: 4)

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00223] 4-1BB ICD (amino acids 198-239 of SEQ ID NO:1; SEQ ID NO: 5)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00224] CD3ζ (amino acids 240-351 of SEQ ID NO: 1; SEQ ID NO: 6)
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00225] **Anti-LAP CAR (H-L) - pMGH 99: CD8 Leader – anti-LAP – CD8 hinge + TM – 4-1BB – CD3z** (SEQ ID NO: 7) comprising CD8 leader sequence (amino acids 1-21 of SEQ ID NO: 7; SEQ ID NO: 8), anti-LAP scFv (H-L) (amino acids 22-307 of SEQ ID NO: 7; SEQ ID NO: 9), CD8 hinge/TM domain (amino acids 308-376 of SEQ ID NO: 7; SEQ ID NO: 10), 4-1BB ICD (amino acids 377-418 of SEQ ID NO: 7; SEQ ID NO: 11), and CD3ζ (amino acids 419-530 of SEQ ID NO: 7; SEQ ID NO: 12).
MALPVTALLLPLALLLHAARPMKLWLNWIFLVTLLNDIQCEVKLVESGGGLVQPG
GSLSLSCAASGFTFTDYYMSWVRQPPGKALEWLGFIRNKPNGYTTEYSASVKG
RFTISRDNSQSILYLQMNVLRAEDSATYYCARYTGGGYFDYWGQGTTLTVSSG
GGGSGGGGSGGGGSGGGGSMMSSAQFLGLLLLCFQGTRCDIQMTQTTSSLS
ASLGDRLTISCRASQDISNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSRFSGSG
SGTDYSLTISNLEQADIATYFCQQGDTLPWTFGGGTKLEIKTTTPAPRPPTPAPT
IASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAP
AYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL
QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
(SEQ ID NO: 7)

[00226] CD8 leader sequence (amino acids 1-21 of SEQ ID NO: 7; SEQ ID NO: 8)
MALPVTALLLPLALLLHAARP

[00227] Anti-LAP scFv (H-L) (amino acids 22-307 of SEQ ID NO: 7; SEQ ID NO: 9)
MKLWLNWIFLVTLLNDIQCEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYM
SWVRQPPGKALEWLGFIRNKPNGYTTEYSASVKGRFTISRDNSQSILYLQMNV
LRAEDSATYYCARYTGGGYFDYWGQGTTLTVSSGGGGSGGGGSGGGGSGGG
GGSMMSSAQFLGLLLLCFQGTRCDIQMTQTTSSLSASLGDRLTISCRASQDISN
YLNWYQQKPDGTVKLLIYYTSRLHSGVPSRFSGSGSGTDYSLTISNLEQADIAT
YFCQQGDTLPWTFGGGTKLEIK

[00228] CD8 hinge/TM domain (amino acids 308-376 of SEQ ID NO: 7; SEQ ID NO: 10)
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00229] 4-1BB ICD (amino acids 377-418 of SEQ ID NO: 7; SEQ ID NO: 11)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00230] CD3ζ (amino acids 419-530 of SEQ ID NO: 7; SEQ ID NO: 12).

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00231] **Anti-LAP CAR (L-H) - pMGH 100: CD8 Leader – anti-LAP – CD8 hinge + TM – 4-1BB – CD3z** (SEQ ID NO: 13) comprising CD8 leader (amino acids 1-21 of SEQ ID NO: 13; SEQ ID NO: 14), anti-LAP scFv (L-H) (amino acids 22-307 of SEQ ID NO: 13; SEQ ID NO: 15), CD8 hinge/TM (amino acids 308-376 of SEQ ID NO: 13; SEQ ID NO: 16), 4-1BB ICD (amino acids 377-418 of SEQ ID NO: 13; SEQ ID NO: 17), and CD3z (amino acids 419-530 of SEQ ID NO: 13; SEQ ID NO: 18).

MALPVTALLLPLALLLHAARPMMSSAQFLGLLLLCFQGTRCDIQMTQTTSSLSA
SLGDRLTISCRASQDISNYLNWYQQKPDGTVKLLIYYTSRLHSGVPSRFSGSGS
GTDYSLTISNLEQADIATYFCQQGDTLPWTFGGGTKLEIKGGGGSGGGGSGGG
GSGGGGSMKLWLNWIFLVTLLNDIQCEVKLVESGGGLVQPGGSLSLSCAASGF
TFTDYYMSWVRQPPGKALEWLGFIRNKPNGYTTEYSASVKGRFTISRDNSQSIL
YLQMNVLRAEDSATYYCARYTGGGYFDYWGQGTTLTVSSTTTPAPRPPTPAP
TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLY
CKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADA
PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNE
LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR
(SEQ ID NO: 13)

[00232] CD8 leader (amino acids 1-21 of SEQ ID NO: 13; SEQ ID NO: 14)

MALPVTALLLPLALLLHAARP

[00233] Anti-LAP scFv (L-H) (amino acids 22-307 of SEQ ID NO: 13; SEQ ID NO: 15)

MMSSAQFLGLLLLCFQGTRCDIQMTQTTSSLSASLGDRLTISCRASQDISNYLN
WYQQKPDGTVKLLIYYTSRLHSGVPSRFSGSGSGTDYSLTISNLEQADIATYFC
QQGDTLPWTFGGGTKLEIKGGGGSGGGGSGGGGSGGGGSMKLWLNWIFLVT
LLNDIQCEVKLVESGGGLVQPGGSLSLSCAASGFTFTDYYMSWVRQPPGKALE
WLGFIRNKPNGYTTEYSASVKGRFTISRDNSQSILYLQMNVLRAEDSATYYCAR
YTGGGYFDYWGQGTTLTVSS

[00234] CD8 hinge/TM (amino acids 308-376 of SEQ ID NO: 13; SEQ ID NO: 16)

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00235] 4-1BB ICD (amino acids 377-418 of SEQ ID NO: 13; SEQ ID NO: 17)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00236] CD3z (amino acids 419-530 of SEQ ID NO: 13; SEQ ID NO: 18)

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00237] **Anti-EGFR CAR secreting anti-GARP Camelid - pMGH 105: CD8 Leader – anti-EGFR – CD8 hinge + TM – 4-1BB – CD3z – anti-GARP camelid** (SEQ ID NO: 19) comprising CD8 leader (amino acids 1-21 of SEQ ID NO: 19; SEQ ID NO: 20), anti-EGFR scFv (amino acids 22-267 of SEQ ID NO: 19; SEQ ID NO: 21), CD8 hinge/TM (amino acids 268-336 of SEQ ID NO: 19; SEQ ID NO: 22), 4-

1BB (amino acids 337-378 of SEQ ID NO: 19; SEQ ID NO: 23), CD3z (amino acids 379-490 of SEQ ID NO: 19; SEQ ID NO: 24), 2A cleavage sequence (amino acids 494-515 of SEQ ID NO: 19; SEQ ID NO: 31), IgK leader (amino acids 519-539 of SEQ ID NO: 19; SEQ ID NO: 32), and anti-GARP camelid (amino acids 540-646 of SEQ ID NO: 19; SEQ ID NO: 25).

MALPVTALLLPLALLLHAARPQVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYG
VHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSL
QSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAGGGGSGGGGSGGGGSGG
GGSDILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYAS
ESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLEL
KTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLA
GTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEG
GCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG
KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD
TYDALHMQALPPRPGSGSGATNFSLLKQAGDVEENPGPRTAMETDTLLLWVLL
LWVPGSTGDDIQMTQSPSSLSASLGDRVTITCQASQSISSYLAWYQQKPGQAP
NILIYGASRLKTGVPSRFSGSGSGTSFTLTISGLEAEDAGTYYCQQYASVPVTFG
QGTKVELKHHHHHHSG (SEQ ID NO: 19)

[00238] CD8 leader (amino acids 1-21 of SEQ ID NO: 19; SEQ ID NO: 20)
MALPVTALLLPLALLLHAARP

[00239] Anti-EGFR scFv (amino acids 22-267 of SEQ ID NO: 19; SEQ ID NO: 21)
QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIW
SGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEF
AYWGQGTLVTVSAGGGGSGGGGSGGGGSGGGGSDILLTQSPVILSVSPGER
VSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTL
SINSVESEDIADYYCQQNNNWPTTFGAGTKLELK

[00240] CD8 hinge/TM (amino acids 268-336 of SEQ ID NO: 19; SEQ ID NO: 22)
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00241] 4-1BB (amino acids 337-378 of SEQ ID NO: 19; SEQ ID NO: 23)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00242] CD3z (amino acids 379-490 of SEQ ID NO: 19; SEQ ID NO: 24)
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00243] 2A cleavage sequence (amino acids 494-515 of SEQ ID NO: 19; SEQ ID NO: 31)
GSGATNFSLLKQAGDVEENPGP

[00244] IgK leader (amino acids 519-539 of SEQ ID NO: 19; SEQ ID NO: 32)
METDTLLLWVLLLWVPGSTGD

[00245] Anti-GARP camelid (amino acids 540-646 of SEQ ID NO: 19; SEQ ID NO: 25).
DIQMTQSPSSLSASLGDRVTITCQASQSISSYLAWYQQKPGQAPNILIYGASRLK
TGVPSRFSGSGSGTSFTLTISGLEAEDAGTYYCQQYASVPVTFGQGTKVELK

[00246] **pMGH113 - 3C10 scFv - CD8 Hinge/TM - 4-1BB ICD - CD3z - P2A - IgK leader - Cetuximab scFv - CD3 scFv - His-tag** (SEQ ID NO: 26) comprising 3C10 scFv (amino acids 1-243 of SEQ ID NO: 26; SEQ ID NO: 27), CD8 hinge/TM (amino acids 244-312 of SEQ ID NO: 26; SEQ ID NO: 28), 4-1BB ICD (amino acids 313-354 of SEQ ID NO: 26; SEQ ID NO: 29), CD3z (amino acids 355-466 of SEQ ID NO: 26; SEQ ID NO: 30), P2A (amino acids 467-488 of SEQ ID NO: 26; SEQ ID NO: 31), IgK leader (amino acids 491-511 of SEQ ID NO: 26; SEQ ID NO: 32), Cetuximab scFv (amino acids 512-752 of SEQ ID NO: 26; SEQ ID NO: 33), CD3 scFv (amino acids 758-1000 of SEQ ID NO: 26; SEQ ID NO: 34).

EIQLQQSGAELVKPGASVKLSCTGSGFNIEDYYIHWVKQRTEQGLEWIGRIDPE
NDETKYGPIFQGRATITADTSSNTVYLQLSSLTSEDTAVYYCAFRGGVYWGPGT
TLTVSSGGGGSGGGGSGGGGSHMDVVMTQSPLTLSVAIGQSASISCKSSQSL
LDSDGKTYLNWLLQRPGQSPKRLISLVSKLDSGVPDRFTGSGSGTDFTLRISRV
EAEDLGIYYCWQGTHFPGTFGGGTKLEIKTTTPAPRPPTPAPTIASQPLSLRPEA
CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFK
QPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLY
NELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYS
EIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATNFSLLKQ
AGDVEENPGPPRMETDTLLLWVLLLWVPGSTGDDILLTQSPVILSVSPGERVSF
SCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSIN
SVESEDIADYYCQQNNNWPTTFGAGTKLELKGGGGSGGGGSGGGGSQVQLK
QSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNT
DYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWG
QGTLVTVSAGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVK
QRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDS
AVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLT
QSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGV
PYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKHHHH
HH (SEQ ID NO: 26)

[00247] 3C10 scFv (amino acids 1-243 of SEQ ID NO: 26; SEQ ID NO: 27)

EIQLQQSGAELVKPGASVKLSCTGSGFNIEDYYIHWVKQRTEQGLEWIGRIDPE
NDETKYGPIFQGRATITADTSSNTVYLQLSSLTSEDTAVYYCAFRGGVYWGPGT
TLTVSSGGGGSGGGGSGGGGSHMDVVMTQSPLTLSVAIGQSASISCKSSQSL
LDSDGKTYLNWLLQRPGQSPKRLISLVSKLDSGVPDRFTGSGSGTDFTLRISRV
EAEDLGIYYCWQGTHFPGTFGGGTKLEIK

[00248] CD8 hinge/TM (amino acids 244-312 of SEQ ID NO: 26; SEQ ID NO: 28)

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00249] 4-1BB ICD (amino acids 313-354 of SEQ ID NO: 26; SEQ ID NO: 29)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00250] CD3z (amino acids 355-466 of SEQ ID NO: 26; SEQ ID NO: 30)

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00251] P2A (amino acids 467-488 of SEQ ID NO: 26; SEQ ID NO: 31)

GSGATNFSLLKQAGDVEENPGP

[00252] IgK leader (amino acids 491-511 of SEQ ID NO: 26; SEQ ID NO: 32)

METDTLLLWVLLLWVPGSTGD

[00253] Cetuximab scFv (amino acids 512-752 of SEQ ID NO: 26; SEQ ID NO: 33)

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESIS
GIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKGG
GGSGGGGSGGGGSQVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVR
QSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTA
IYYCARALTYYDYEFAYWGQGTLVTVSA

[00254] CD3 scFv (amino acids 758-1000 of SEQ ID NO: 26; SEQ ID NO: 34)

DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINP
SRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEKVTM
TCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTI
SSMEAEDAATYYCQQWSSNPLTFGAGTKLELK

[00255] **pMGH133 - 2173 scFv - CD8 Hinge/TM - 4-1BB ICD - CD3z - P2A - IgK leader - Cetuximab scFv - CD3 scFv - His-tag** (SEQ ID NO: 35) comprising 2173 scFv (amino acids 1-246 of SEQ ID NO: 35; SEQ ID NO: 36), CD8 hinge/TM (amino acids 247-315 of SEQ ID NO: 35; SEQ ID NO: 37), 4-1BB ICD (amino acids 316-357 of SEQ ID NO: 36; SEQ ID NO: 38), CD3z (amino acids 358-469 of SEQ ID NO: 35; SEQ ID NO: 39), P2A (amino acids 470-491 of SEQ ID NO: 35; SEQ ID NO: 40), IgK leader (amino acids 494-514 of SEQ ID NO: 35; SEQ ID NO: 41), Cetuximab scFv (amino acids 515-755 of SEQ ID NO: 35; SEQ ID NO: 42), and CD3 scFv (amino acids 761-1003 of SEQ ID NO: 35; SEQ ID NO: 43).

EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIKTTTPAPRPPTPAPTIASQ
PLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR
KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ
GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATN
FSLLKQAGDVEENPGPPRMETDTLLLWVLLLWVPGSTGDDILLTQSPVILSVSP
GERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGT
DFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKGGGGSGGGGSGGGG
SQVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVI
WSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDY

EFAYWGQGTLVTVSAGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRY
TMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLS
SLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGG
VDDIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTS
KVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLE
LKHHHHHH (SEQ ID NO: 35)

[00256] 2173 scFv (amino acids 1-246 of SEQ ID NO: 35; SEQ ID NO: 36)
EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIK

[00257] CD8 hinge/TM (amino acids 247-315 of SEQ ID NO: 35; SEQ ID NO: 37)
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00258] 4-1BB ICD (amino acids 316-357 of SEQ ID NO: 35; SEQ ID NO: 38)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00259] CD3z (amino acids 358-469 of SEQ ID NO: 35; SEQ ID NO: 39)
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00260] P2A (amino acids 470-491 of SEQ ID NO: 35; SEQ ID NO: 40)
GSGATNFSLLKQAGDVEENPGP

[00261] IgK leader (amino acids 494-514 of SEQ ID NO: 35; SEQ ID NO: 41)
METDTLLLWVLLLWVPGSTGD

[00262] Cetuximab scFv (amino acids 515-755 of SEQ ID NO: 35; SEQ ID NO: 42)
DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESIS
GIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKGG
GGSGGGGSGGGGSQVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVR
QSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTA
IYYCARALTYYDYEFAYWGQGTLVTVSA

[00263] CD3 scFv (amino acids 761-1003 of SEQ ID NO: 35; SEQ ID NO: 43)
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINP
SRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEKVTM
TCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTI
SSMEAEDAATYYCQQWSSNPLTFGAGTKLELK

[00264] **pMGH134 - 2173 scFv - CD8 Hinge/TM - 4-1BB ICD - CD3z - P2A - IgK leader - CD19 scFv - CD3 scFv - His-tag** (SEQ ID NO: 44) comprising 2173 scFv (amino acids 1-246 of SEQ ID NO: 44; SEQ ID NO: 45), CD8 hinge/TM (amino acids 247-315 of SEQ ID NO: 44; SEQ ID NO: 46), 4-1BB ICD (amino acids 316-357 of SEQ ID NO: 44; SEQ ID NO: 47), CD3z (amino acids 358-469 of SEQ ID

NO: 44; SEQ ID NO: 48), P2A (amino acids 470-491 of SEQ ID NO: 44; SEQ ID NO: 49), IgK leader (amino acids 494-514 of SEQ ID NO: 44; SEQ ID NO: 50), CD19 scFv (amino acids 515-764 of SEQ ID NO: 44; SEQ ID NO: 51), CD3 scFv (amino acids 770-1012 of SEQ ID NO: 44; SEQ ID NO: 52).

EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIKTTTPAPRPPTPAPTIASQ
PLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR
KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ
GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGATN
FSLLKQAGDVEENPGPPRMETDTLLLWVLLLWVPGSTGDDIQLTQSPASLAVSL
GQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGS
GSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTKLEIKGGGGSGGGGS
GGGGSQVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLE
WIGQIWPGDGDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARR
ETTTVGRYYYAMDYWGQGTTVTVSSGGGGSDIKLQQSGAELARPGASVKMSC
KTSGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKS
SSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGS
GGSGGSGGVDDIQLTQSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTS
PKRWIYDTSKVASGVPYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPL
TFGAGTKLELKHHHHHH (SEQ ID NO: 44)

[00265] 2173 scFv (amino acids 1-246 of SEQ ID NO: 44; SEQ ID NO: 45)

EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIK

[00266] CD8 hinge/TM (amino acids 247-315 of SEQ ID NO: 44; SEQ ID NO: 46)

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00267] 4-1BB ICD (amino acids 316-357 of SEQ ID NO: 44; SEQ ID NO: 47)

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00268] CD3z (amino acids 358-469 of SEQ ID NO: 44; SEQ ID NO: 48)

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK
NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH
MQALPPR

[00269] P2A (amino acids 470-491 of SEQ ID NO: 44; SEQ ID NO: 49)

GSGATNFSLLKQAGDVEENPGP

[00270] IgK leader (amino acids 494-514 of SEQ ID NO: 44; SEQ ID NO: 50)

METDTLLLWVLLLWVPGSTGD

[00271] CD19 scFv (amino acids 515-764 of SEQ ID NO: 44; SEQ ID NO: 51)

DIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYD
ASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTK
LEIKGGGGSGGGGSGGGGSQVQLQQSGAELVRPGSSVKISCKASGYAFSSY
WMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTADESSSTAYMQL
SSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTTVTVSS

[00272] CD3 scFv (amino acids 770-1012 of SEQ ID NO: 44; SEQ ID NO: 52)

DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINP
SRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEKVTM
TCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTI
SSMEAEDAATYYCQQWSSNPLTFGAGTKLELK

[00273] **pMGH135 - (NFAT response element) - IgK leader - Cetuximab scFv - CD3 scFv - His-tag - (EF1a Promoter) - 2173 scFv - CD8 hinge/TM - 4-1BB ICD - CD3z** (SEQ ID NO: 53) comprising IgK leader (amino acids 1-21 of SEQ ID NO: 53; SEQ ID NO: 54), Cetuximab scFv (amino acids 22-262 of SEQ ID NO: 53; SEQ ID NO: 55), CD3 scFv (amino acids 268-510 of SEQ ID NO: 53; SEQ ID NO: 56), 2173 scFv (amino acids 517-762 of SEQ ID NO: 53; SEQ ID NO: 57), CD8 hinge/TM (amino 763-831 of SEQ ID NO: 53; SEQ ID NO: 58), 4-1BB ICD (amino acids 832-873 of SEQ ID NO: 53; SEQ ID NO: 59), CD3z (amino acids 874-985 of SEQ ID NO: 53; SEQ ID NO: 60).

(NFAT response element)
METDTLLLWVLLLWVPGSTGDDILLTQSPVILSVSPGERVSFSCRASQSIGTNIH
WYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQ
QNNNWPTTFGAGTKLELKGGGGSGGGGSGGGGSQVQLKQSGPGLVQPSQS
LSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDYNTPFTSRLSIN
KDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSAGGG
GSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGY
INPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDH
YCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEK
VTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSY
SLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKHHHHHH
(EF1a Promoter)
EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIKTTTPAPRPPTPAPTIASQ
PLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR
KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ
GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID

NO: 53) (Note: the two polypeptides noted above are denoted with a single sequence identifier for convenience, but it should be understood that the CAR and BiTE components can be made separately, due to the two separate promoters; see above.)

[00274] IgK leader (amino acids 1-21 of SEQ ID NO: 53; SEQ ID NO: 54)
METDTLLLWVLLLWVPGSTGD

[00275] Cetuximab scFv (amino acids 22-262 of SEQ ID NO: 53; SEQ ID NO: 55)
DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESIS
GIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELKGG
GGSGGGGSGGGGSQVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVR
QSPGKGLEWLGVIWSGGNTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDTA
IYYCARALTYYDYEFAYWGQGTLVTVSA

[00276] CD3 scFv (amino acids 268-510 of SEQ ID NO: 53; SEQ ID NO: 56)
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINP
SRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEKVTM
TCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTI
SSMEAEDAATYYCQQWSSNPLTFGAGTKLELK

[00277] 2173 scFv (amino acids 517-762 of SEQ ID NO: 53; SEQ ID NO: 57)
EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIK

[00278] CD8 hinge/TM (amino acids 763-831 of SEQ ID NO: 53; SEQ ID NO: 58)
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00279] 4-1BB ICD (amino acids 832-873 of SEQ ID NO: 53; SEQ ID NO: 59)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00280] CD3z (amino acids 874-985 of SEQ ID NO: 53; SEQ ID NO: 60)
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00281] **pMGH136 - (NFAT response element) - IgK leader - CD19 scFv - CD3 scFv - His-tag - (EF1a Promoter) - 2173 scFv - CD8 Hinge/TM - 4-1BB ICD - CD3z** (SEQ ID NO:61) comprising (NFAT response element), IgK leader (amino acids 1-21 of SEQ ID NO: 61; SEQ ID NO: 62), CD19 scFv (amino acids 22-271 of SEQ ID NO: 61; SEQ ID NO: 63), CD3 scFv (amino acids 277-519 of SEQ ID NO: 61; SEQ ID NO: 64), 2173 scFv (amino acids 526-771 of SEQ ID NO: 61; SEQ ID NO: 65), CD8 hinge/TM (amino acids 772-840 of SEQ ID NO: 61; SEQ ID NO: 66), 4-1BB ICD (amino acids 841-882 of SEQ ID NO: 61; SEQ ID NO: 67), CD3z (amino acids 883-994 of SEQ ID NO: 61; SEQ ID NO: 68).

METDTLLLWVLLLWVPGSTGDDIQLTQSPASLAVSLGQRATISCKASQSVDYDG
DSYLNWYQQIPGQPPKLLIYDASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDA
ATYHCQQSTEDPWTFGGGTKLEIKGGGGSGGGGSGGGGSQVQLQQSGAELV
RPGSSVKISCKASGYAFSSYWMNWVKQRPGQGLEWIGQIWPGDGDTNYNGK
FKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYYAMDYWG
QGTTVTVSSGGGGSDIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVK
QRPGQGLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDS
AVYYCARYYDDHYCLDYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLT
QSPAIMSASPGEKVTMTCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGV
PYRFSGSGSGTSYSLTISSMEAEDAATYYCQQWSSNPLTFGAGTKLELKHHHH
HH

(EF1a Promoter)

EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIKTTTPAPRPPTPAPTIASQ
PLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGR
KKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ
GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR (SEQ ID NO: 61) (Note: the two polypeptides noted above are denoted with a single sequence identifier for convenience, but it should be understood that the CAR and BiTE components can be made separately, due to the two separate promoters; see above.)

[00282] IgK leader (amino acids 1-21 of SEQ ID NO: 61; SEQ ID NO: 62)
METDTLLLWVLLLWVPGSTGD

[00283] CD19 scFv (amino acids 22-271 of SEQ ID NO: 61; SEQ ID NO: 63)
DIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYD
ASNLVSGIPPRFSGSGSGTDFTLNIHPVEKVDAATYHCQQSTEDPWTFGGGTK
LEIKGGGGSGGGGSGGGGSQVQLQQSGAELVRPGSSVKISCKASGYAFSSY
WMNWVKQRPGQGLEWIGQIWPGDGDTNYNGKFKGKATLTADESSSTAYMQL
SSLASEDSAVYFCARRETTTVGRYYYAMDYWGQGTTVTVSS

[00284] CD3 scFv (amino acids 277-519 of SEQ ID NO: 61; SEQ ID NO: 64)
DIKLQQSGAELARPGASVKMSCKTSGYTFTRYTMHWVKQRPGQGLEWIGYINP
SRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCL
DYWGQGTTLTVSSVEGGSGGSGGSGGSGGVDDIQLTQSPAIMSASPGEKVTM
TCRASSSVSYMNWYQQKSGTSPKRWIYDTSKVASGVPYRFSGSGSGTSYSLTI
SSMEAEDAATYYCQQWSSNPLTFGAGTKLELK

[00285] 2173 scFv (amino acids 526-771 of SEQ ID NO: 61; SEQ ID NO: 65)

EIQLVQSGAEVKKPGESLRISCKGSGFNIEDYYIHWVRQMPGKGLEWMGRIDP
ENDETKYGPIFQGHVTISADTSINTVYLQWSSLKASDTAMYYCAFRGGVYWGQ
GTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQSPDSLAVSLGERATINC
KSSQSLLDSDGKTYLNWLQQKPGQPPKRLISLVSKLDSGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCWQGTHFPGTFGGGTKVEIK

[00286] CD8 hinge/TM (amino acids 772-840 of SEQ ID NO: 61; SEQ ID NO: 66)
TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT
CGVLLLSLVITLYC

[00287] 4-1BB ICD (amino acids 841-882 of SEQ ID NO: 61; SEQ ID NO: 67)
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

[00288] CD3z (amino acids 883-994 of SEQ ID NO: 61; SEQ ID NO: 68)
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR
KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA
LHMQALPPR

[00289] Other aspects of the invention are within the scope of the following numbered paragraphs.

1. A chimeric antigen receptor (CAR) T cell comprising a heterologous nucleic acid molecule, wherein the heterologous nucleic acid molecule comprises:

(a) a first polynucleotide encoding a CAR comprising an antigen-binding domain, a transmembrane domain, and an intracellular signaling domain; and

(b) a second polynucleotide encoding a therapeutic agent.

2. The CAR T cell of paragraph 1, wherein the therapeutic agent comprises an antibody reagent.

3. The CAR T cell of paragraph 2, wherein the antibody reagent comprises a single chain antibody or a single domain antibody.

4. The CAR T cell of paragraph 2 or 3, wherein the antibody reagent comprises a bispecific antibody reagent.

5. The CAR T cell of paragraph 4, wherein the bispecific antibody reagent comprises a bispecific T cell engager (BiTE).

6. The CAR T cell of paragraph 3, wherein the single domain antibody comprises a camelid antibody.

7. The CAR T cell of paragraph 1, wherein the therapeutic agent comprises a cytokine.

8. The CAR T cell of any one of paragraphs 1 to 7, wherein the CAR and the therapeutic agent are produced in the form of a polyprotein, which is cleaved to generate separate CAR and therapeutic agent molecules.

9. The CAR T cell of paragraph 8, wherein the polyprotein comprises a cleavable moiety between the CAR and the therapeutic agent.

10. The CAR T cell of paragraph 9, wherein the cleavable moiety comprises a 2A peptide.

11. The CAR T cell of paragraph 10, wherein the 2A peptide comprises P2A or T2A.

12. The CAR T cell of any one of paragraphs 1 to 11, wherein the CAR and the therapeutic agent are each constitutively expressed.

13. The CAR T cell of any one of paragraphs 1 to 12, wherein expression of the CAR and the therapeutic agent is driven by an elongation factor-1 alpha (EF1α) promoter.

14. The CAR T cell of any one of paragraphs 1 to 11, wherein the therapeutic agent is expressed under the control of an inducible promoter, which is optionally inducible by T cell receptor or CAR signaling.

15. The CAR T cell of paragraph 14, wherein the inducible promoter comprises the NFAT promoter.

16. The CAR T cell of any one of paragraphs 1 to 11, wherein the CAR is expressed under the control of a constitutive promoter and the therapeutic agent is expressed under the control of an inducible promoter, which is optionally inducible by T cell receptor or CAR signaling.

17. The CAR T cell of any one of paragraph 1 to 16, wherein the CAR further comprises one or more co-stimulatory domains.

18. The CAR T cell of any one of paragraphs 1 to 17, wherein the antigen-binding domain of the CAR comprises an antibody, a single chain antibody, a single domain antibody, or a ligand.

19. The CAR T cell of any one of paragraphs 1 to 18, wherein the transmembrane domain of the CAR comprises a CD8 hinge/transmembrane domain, which optionally comprises the sequence of any one of SEQ ID NOs: 4, 10, 16, 22, 28, 37, 46, 58, and 66, or a variant thereof.

20. The CAR T cell of any one of paragraphs 1 to 19, wherein the intracellular signaling domain comprises a CD3ζ intracellular signaling domain, which optionally comprises the sequence of any one of SEQ ID NOs: 6, 12, 18, 24, 30, 39, 48, 60, and 68, or a variant thereof.

21. The CAR T cell of any one of paragraphs 1 to 20, comprising a 4-1BB co-stimulatory domain, which optionally comprises the sequence of any one of SEQ ID NOs: 5, 11, 17, 23, 29, 38, 47, 59, and 67, or a variant thereof.

22. The CAR T cell of any one of paragraphs 1-21, wherein the CAR antigen-binding domain or the therapeutic agent, when the therapeutic agent comprises an antibody reagent, bind to a tumor-associated antigen.

23. The CAR T cell of paragraph 22, wherein the tumor-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds is a solid tumor-associated antigen.

24. The CAR T cell of paragraph 22 or 23, wherein the tumor-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds comprises epidermal growth factor receptor variant III (EGFRvIII), EGFR, CD19, prostate-specific membrane antigen (PSMA), or IL-13 receptor alpha 2 (IL-13Rα2), and optionally the CAR antigen-binding domain or the therapeutic agent comprises a sequence selected from the group consisting of SEQ ID NO: 21, 27, 33, 36, 42, 45, 51, 55, 57, 63, 65, and variants thereof.

25. The CAR T cell of any one of paragraphs 1 to 21, wherein the CAR antigen-binding domain or the therapeutic agent, when the therapeutic agent comprises an antibody reagent, binds to a Treg-associated antigen.

26. The CAR T cell of paragraph 25, wherein the Treg-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds is selected from the group consisting of glycoprotein A repetitions predominant (GARP), latency-associated peptide (LAP), CD25, and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and optionally the CAR antigen-binding

domain or the therapeutic agent comprises a sequence selected from the group consisting of SEQ ID NO: 3, 9, 15, 25, and variants thereof.

27. A CAR T cell comprising a polynucleotide encoding a CAR, wherein the CAR comprises an antigen-binding domain, a transmembrane domain, and an intracellular signaling domain; and the antigen-binding domain binds to a Treg-associated antigen.

28. The CAR T cell of paragraph 27, wherein the Treg-associated antigen is selected from the group consisting of GARP, LAP, CD25, and CTLA-4.

29. The CAR T cell of paragraph 27 or 28, wherein the CAR further comprises one or more co-stimulatory domains.

30. The CAR T cell of any one of paragraphs 27 to 29, wherein the antigen-binding domain of the CAR comprises a scFv or a single domain antibody, which optionally comprises a sequence selected from the group consisting of SEQ ID NO: 3, 9, 15, 25, and variants thereof.

31. A CAR T cell comprising a heterologous nucleic acid molecule encoding an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NO: 26, SEQ ID NO: 35, SEQ ID NO: 44, SEQ ID NO: 53, SEQ ID NO: 61, SEQ ID NO: 19, SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13.

32. The CAR T cell of paragraph 31, comprising a heterologous nucleic acid molecule encoding an amino acid sequence of any one of SEQ ID NO: 26, SEQ ID NO: 35, SEQ ID NO: 44, SEQ ID NO: 53, SEQ ID NO: 61, SEQ ID NO: 19, SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13.

33. A nucleic acid molecule encoding (i) a CAR polypeptide, or (ii) a polyprotein comprising a CAR polypeptide and a therapeutic agent, of any one of paragraphs 1 to 32.

34. A CAR polypeptide, or polyprotein comprising a CAR polypeptide and a therapeutic agent, of any one of paragraphs 1 to 32.

35. A pharmaceutical composition comprising one or more CAR T cells, nucleic acid molecules, CAR polypeptides, or a polyproteins of any one of paragraphs 1 to 34.

36. A method of treating a patient having cancer, the method comprising administering to the patient a pharmaceutical composition comprising one or more CAR T cell of any one of paragraphs 1-32 or a pharmaceutical composition of paragraph 35.

37. The method of paragraph 36, wherein by targeting the tumor microenvironment, systemic toxicity is reduced.

38. The method of paragraph 36 or 37, wherein the cancer is characterized by the presence of one or more solid tumors.

39. The method of any one of paragraphs 36 to 38, wherein the cancer is characterized by tumor-infiltrating Tregs.

40. The method of any one of paragraphs 36 to 39, wherein the cancer is a glioblastoma.

41. A method of treating a patient having cancer, the method comprising administering to the patient a CAR T cell product, genetically modified to secrete a tumor-toxic antibody or cytokine, wherein by directing the cancer toxicity locally to the tumor microenvironment, systemic toxicity is reduced.

42. The method of paragraph 41, wherein the CAR T cell is genetically modified to deliver an antibody against CTLA4, CD25, GARP, LAP, IL15, CSF1R, or EGFR, or a bispecific antibody against to the tumor microenvironment.

43. The method of paragraph 42, wherein the bispecific antibody is directed against EGFR and CD3.

44. A method of delivering a therapeutic agent to a tissue or organ in a patient to treat a disease or pathology, the method comprising administering to said patient a CAR T cell, genetically modified to secrete a therapeutic antibody, toxin, or agent, wherein the therapeutic antibody, toxin, or agent would, by itself, be unable to enter or penetrate the tissue or organ.

45. The method of paragraph 44, wherein the tissue or organ is in the nervous system.

46. The method of paragraph 45, wherein the nervous system is the central nervous system.

47. The method of paragraph 46, wherein the central nervous system is the brain.

48. The method of any one of paragraphs 44 to 47, wherein the disease or pathology is glioblastoma.

49. The method of paragraph 44, wherein the therapeutic antibody is anti-EGFR (anti-epidermal growth factor receptor) or anti-EGFRvIII.

[00290] The following claims are meant to be representative only and not to limit the scope of the disclosed invention. In at least one aspect, we claim:

What is claimed is:

1. A chimeric antigen receptor (CAR) T cell comprising a heterologous nucleic acid molecule, wherein the heterologous nucleic acid molecule comprises:

(a) a first polynucleotide encoding a CAR comprising an antigen-binding domain, a transmembrane domain, and an intracellular signaling domain; and

(b) a second polynucleotide encoding a therapeutic agent.

2. The CAR T cell of claim 1, wherein the therapeutic agent comprises an antibody reagent.

3. The CAR T cell of claim 2, wherein the antibody reagent comprises a single chain antibody or a single domain antibody.

4. The CAR T cell of claim 2, wherein the antibody reagent comprises a bispecific antibody reagent.

5. The CAR T cell of claim 4, wherein the bispecific antibody reagent comprises a bispecific T cell engager (BiTE).

6. The CAR T cell of claim 3, wherein the single domain antibody comprises a camelid antibody.

7. The CAR T cell of claim 1, wherein the therapeutic agent comprises a cytokine.

8. The CAR T cell of claim 1, wherein the CAR and the therapeutic agent are produced in the form of a polyprotein, which is cleaved to generate separate CAR and therapeutic agent molecules.

9. The CAR T cell of claim 8, wherein the polyprotein comprises a cleavable moiety between the CAR and the therapeutic agent.

10. The CAR T cell of claim 9, wherein the cleavable moiety comprises a 2A peptide.

11. The CAR T cell of claim 10, wherein the 2A peptide comprises P2A or T2A.

12. The CAR T cell of claim 1, wherein the CAR and the therapeutic agent are each constitutively expressed.

13. The CAR T cell of claim 1, wherein expression of the CAR and the therapeutic agent is driven by an elongation factor-1 alpha (EF1α) promoter.

14. The CAR T cell of claim 1, wherein the therapeutic agent is expressed under the control of an inducible promoter, which is optionally inducible by T cell receptor or CAR signaling.

15. The CAR T cell of claim 14, wherein the inducible promoter comprises the NFAT promoter.

16. The CAR T cell of claim 1, wherein the CAR is expressed under the control of a constitutive promoter and the therapeutic agent is expressed under the control of an inducible promoter, which is optionally inducible by T cell receptor or CAR signaling.

17. The CAR T cell of claim 1, wherein the CAR further comprises one or more co-stimulatory domains.

18. The CAR T cell of claim 1, wherein the antigen-binding domain of the CAR comprises an antibody, a single chain antibody, a single domain antibody, or a ligand.

19. The CAR T cell of claim 1, wherein the transmembrane domain of the CAR comprises a CD8 hinge/transmembrane domain, which optionally comprises the sequence of any one of SEQ ID NOs: 4, 10, 16, 22, 28, 37, 46, 58, and 66, or a variant thereof.

20. The CAR T cell of claim 1, wherein the intracellular signaling domain comprises a CD3ζ intracellular signaling domain, which optionally comprises the sequence of any one of SEQ ID NOs: 6, 12, 18, 24, 30, 39, 48, 60, and 68, or a variant thereof.

21. The CAR T cell of claim 1, comprising a 4-1BB co-stimulatory domain, which optionally comprises the sequence of any one of SEQ ID NOs: 5, 11, 17, 23, 29, 38, 47, 59, and 67, or a variant thereof.

22. The CAR T cell of claim 1, wherein the CAR antigen-binding domain or the therapeutic agent, when the therapeutic agent comprises an antibody reagent, bind to a tumor-associated antigen.

23. The CAR T cell of claim 22, wherein the tumor-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds is a solid tumor-associated antigen.

24. The CAR T cell of claim 22, wherein the tumor-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds comprises epidermal growth factor receptor variant III (EGFRvIII), EGFR, CD19, prostate-specific membrane antigen (PSMA), or IL-13 receptor alpha 2 (IL-13Rα2), and optionally the CAR antigen-binding domain or the therapeutic agent comprises a sequence selected from the group consisting of SEQ ID NO: 21, 27, 33, 36, 42, 45, 51, 55, 57, 63, 65, and variants thereof.

25. The CAR T cell of claim 1, wherein the CAR antigen-binding domain or the therapeutic agent, when the therapeutic agent comprises an antibody reagent, binds to a Treg-associated antigen.

26. The CAR T cell of claim 25, wherein the Treg-associated antigen to which the CAR antigen-binding domain or the therapeutic agent binds is selected from the group consisting of glycoprotein A repetitions predominant (GARP), latency-associated peptide (LAP), CD25, and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and optionally the CAR antigen-binding domain or the therapeutic agent comprises a sequence selected from the group consisting of SEQ ID NO: 3, 9, 15, 25, and variants thereof.

27. A CAR T cell comprising a polynucleotide encoding a CAR, wherein the CAR comprises an antigen-binding domain, a transmembrane domain, and an intracellular signaling domain; and the antigen-binding domain binds to a Treg-associated antigen.

28. The CAR T cell of claim 27, wherein the Treg-associated antigen is selected from the group consisting of GARP, LAP, CD25, and CTLA-4.

29. The CAR T cell of claim 27, wherein the CAR further comprises one or more co-stimulatory domains.

30. The CAR T cell of claim 27, wherein the antigen-binding domain of the CAR comprises a scFv or a single domain antibody, which optionally comprises a sequence selected from the group consisting of SEQ ID NO: 3, 9, 15, 25, and variants thereof.

31. A CAR T cell comprising a heterologous nucleic acid molecule encoding an amino acid sequence having at least 90% sequence identity to the amino acid sequence of any one of SEQ ID NO: 26, SEQ ID NO: 35, SEQ ID NO: 44, SEQ ID NO: 53, SEQ ID NO: 61, SEQ ID NO: 19, SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13.

32. The CAR T cell of claim 31, comprising a heterologous nucleic acid molecule encoding an amino acid sequence of any one of SEQ ID NO: 26, SEQ ID NO: 35, SEQ ID NO: 44, SEQ ID NO: 53, SEQ ID NO: 61, SEQ ID NO: 19, SEQ ID NO: 1, SEQ ID NO: 7, and SEQ ID NO: 13.

33. A nucleic acid molecule encoding (i) a CAR polypeptide, or (ii) a polyprotein comprising a CAR polypeptide and a therapeutic agent, of claim 1.

34. A CAR polypeptide, or polyprotein comprising a CAR polypeptide and a therapeutic agent, of claim 1.

35. A pharmaceutical composition comprising one or more CAR T cells, nucleic acid molecules, CAR polypeptides, or a polyproteins of claim 1.

36. A method of treating a patient having cancer, the method comprising administering to the patient a pharmaceutical composition comprising one or more CAR T cell of claim 1 or a nucleic acid molecule, CAR polypeptide, or polyprotein thereof.

37. The method of claim 36, wherein by targeting the tumor microenvironment, systemic toxicity is reduced.

38. The method of claim 36, wherein the cancer is characterized by the presence of one or more solid tumors.

39. The method of claim 36, wherein the cancer is characterized by tumor-infiltrating Tregs.

40. The method of claim 36, wherein the cancer is a glioblastoma.

41. A method of treating a patient having cancer, the method comprising administering to the patient a CAR T cell product, genetically modified to secrete a tumor-toxic antibody or cytokine, wherein by directing the cancer toxicity locally to the tumor microenvironment, systemic toxicity is reduced.

42. The method of claim 41, wherein the CAR T cell is genetically modified to deliver an antibody against CTLA4, CD25, GARP, LAP, IL15, CSF1R, or EGFR, or a bispecific antibody against to the tumor microenvironment.

43. The method of claim 42, wherein the bispecific antibody is directed against EGFR and CD3.

44. A method of delivering a therapeutic agent to a tissue or organ in a patient to treat a disease or pathology, the method comprising administering to said patient a CAR T cell, genetically modified to secrete a therapeutic antibody, toxin, or agent, wherein the therapeutic antibody, toxin, or agent would, by itself, be unable to enter or penetrate the tissue or organ.

45. The method of claim 44, wherein the tissue or organ is in the nervous system.

46. The method of claim 45, wherein the nervous system is the central nervous system.

47. The method of claim 46, wherein the central nervous system is the brain.

48. The method of claims 44, wherein the disease or pathology is glioblastoma.

49. The method of claim 44, wherein the therapeutic antibody is anti-EGFR (anti-epidermal growth factor receptor) or anti-EGFRvIII.

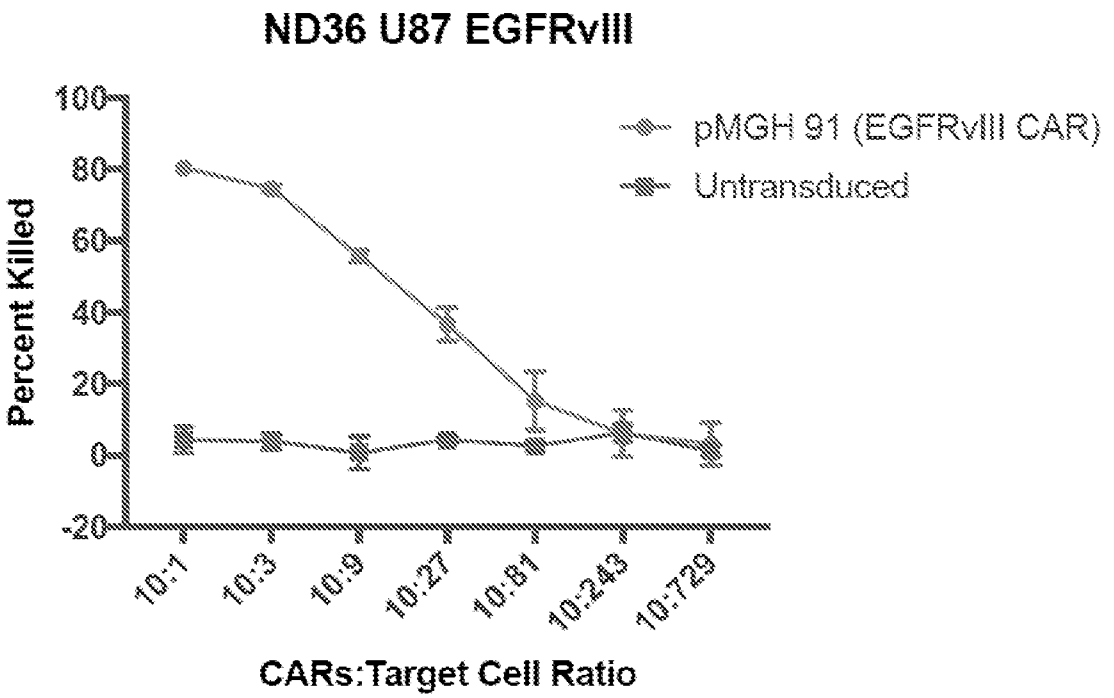

FIG. 1

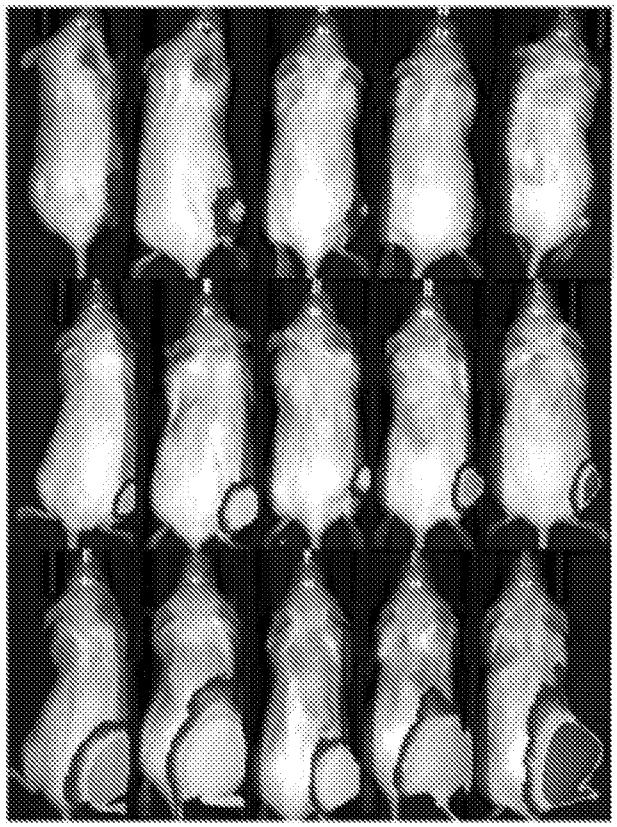

FIG. 2A

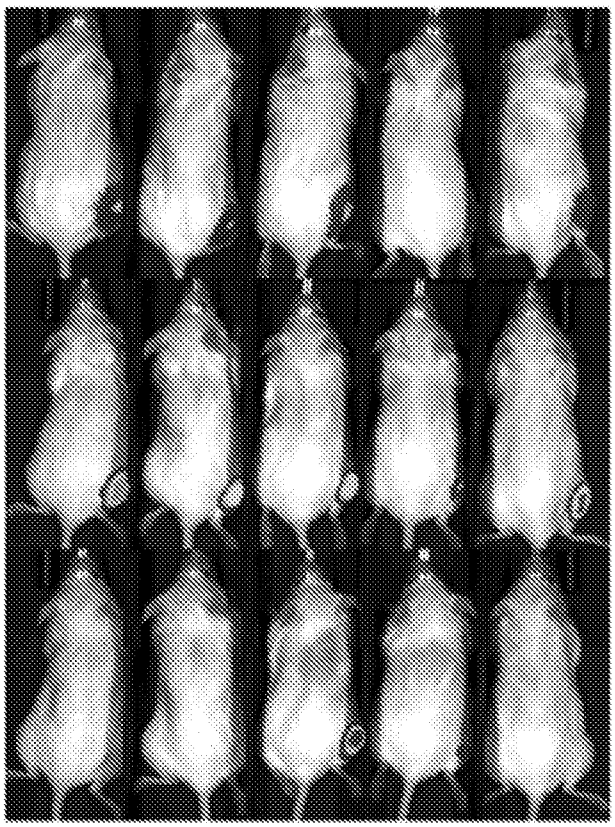

FIG. 2B

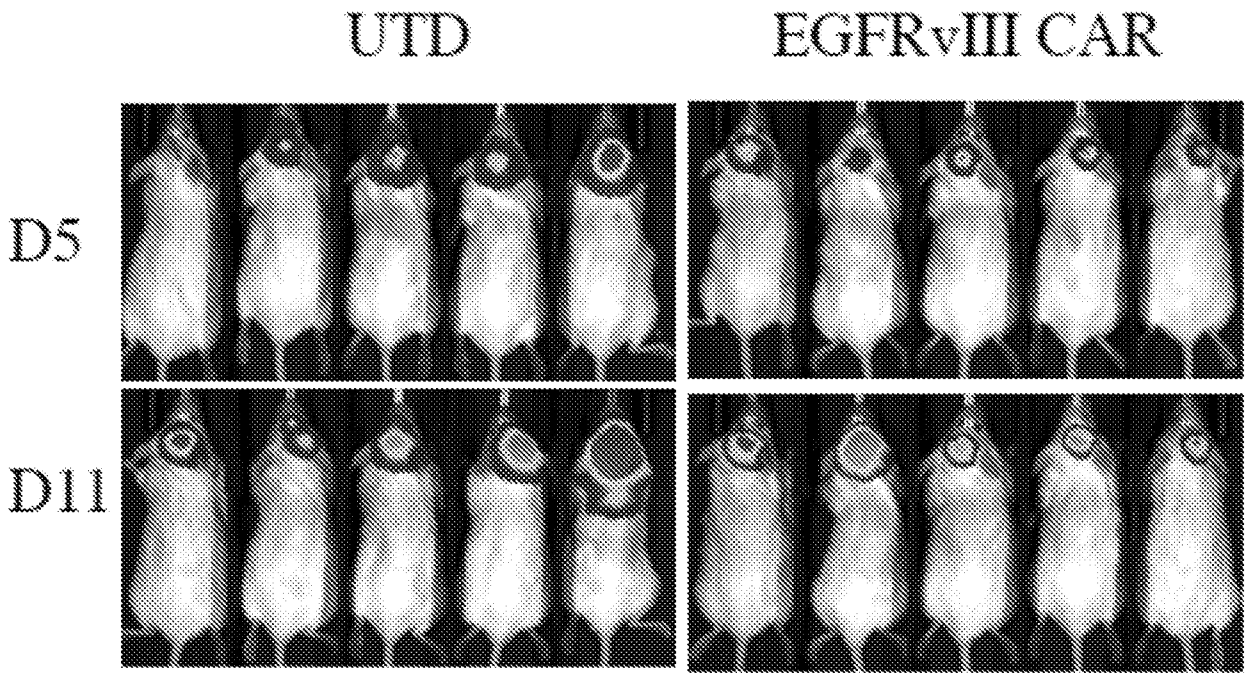

FIG. 3A FIG. 3B

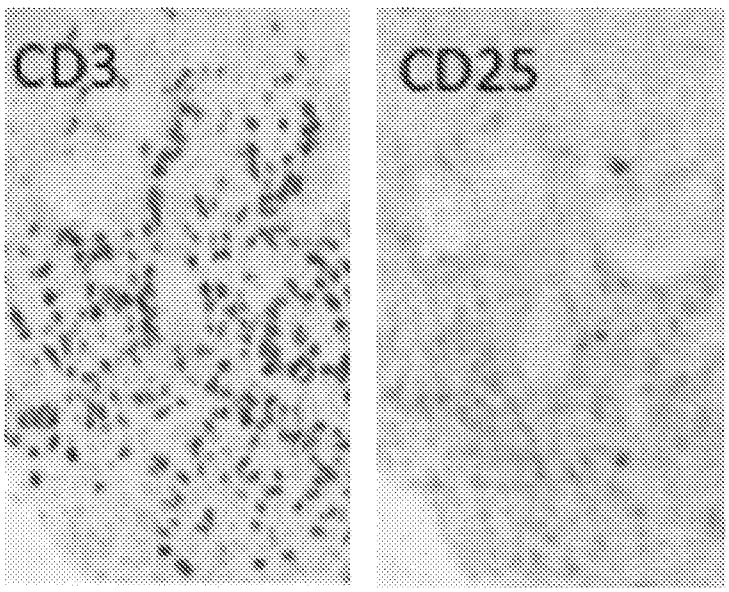

FIG. 4A FIG. 4B

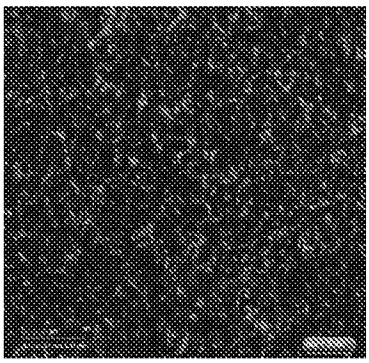

FIG. 5A

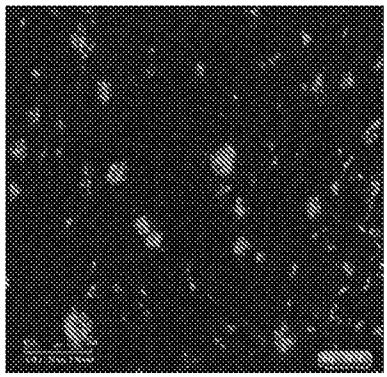

FIG. 5B

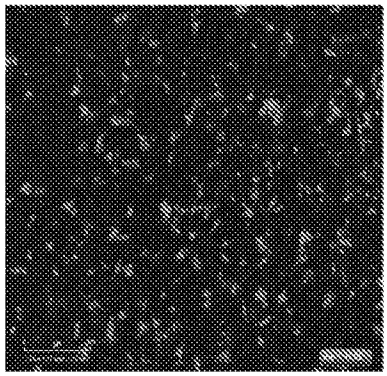

FIG. 5C

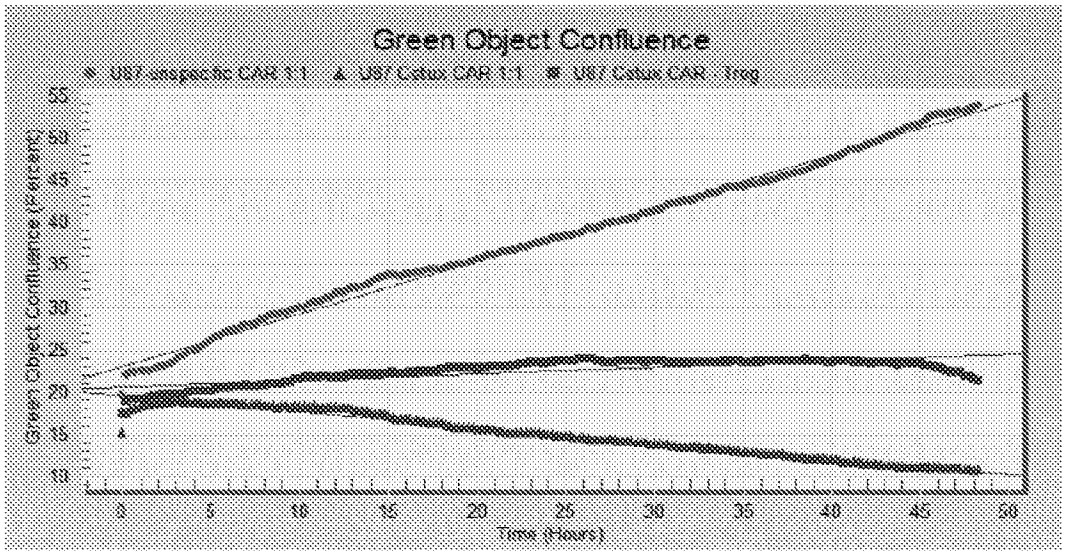

FIG. 5D

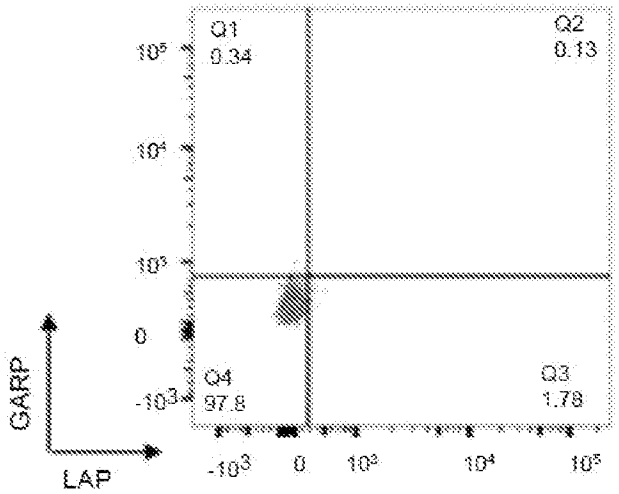
Q1
0.34
Q2
0.13
Q4
97.8
Q3
1.78
GARP
LAP
10^5
10^4
10^3
0
-10^3
-10^3
0
10^3
10^4
10^5

FIG. 6A

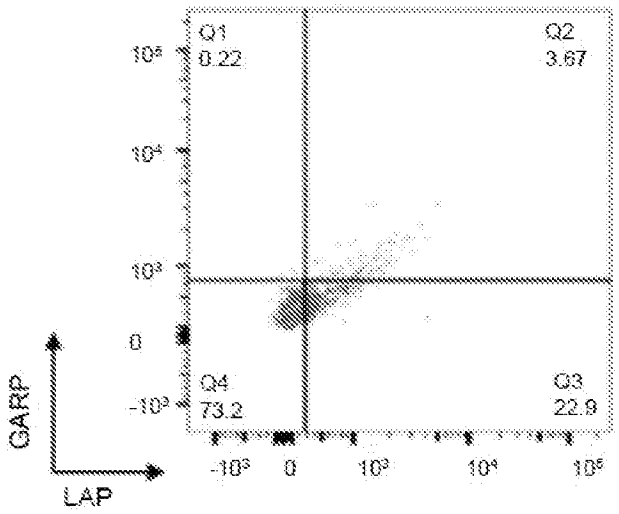
Q1
0.22
Q2
3.67
Q4
73.2
Q3
22.9
GARP
LAP
10^5
10^4
10^3
0
-10^3
-10^3
0
10^3
10^4
10^5

FIG. 6B

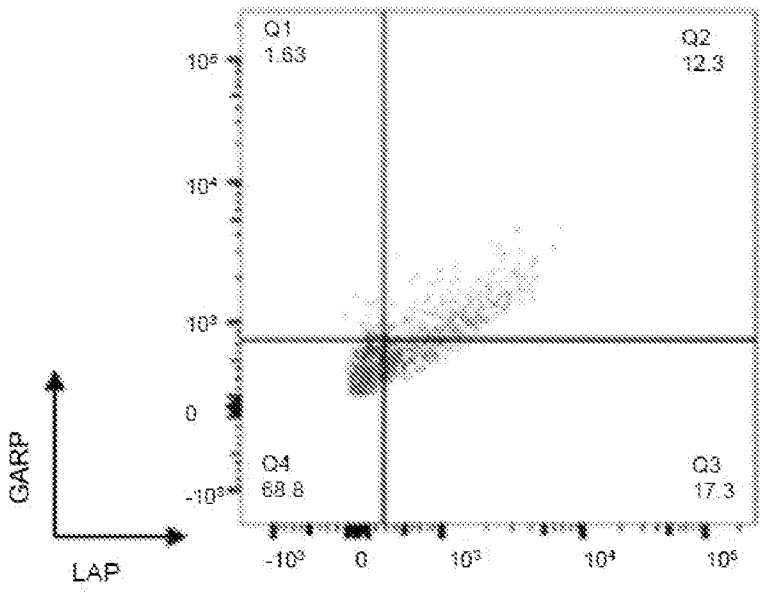
Q1
1.63
Q2
12.3
Q4
68.8
Q3
17.3
GARP
LAP
10^5
10^4
10^3
0
-10^3
-10^3
0
10^3
10^4
10^5

FIG. 6C

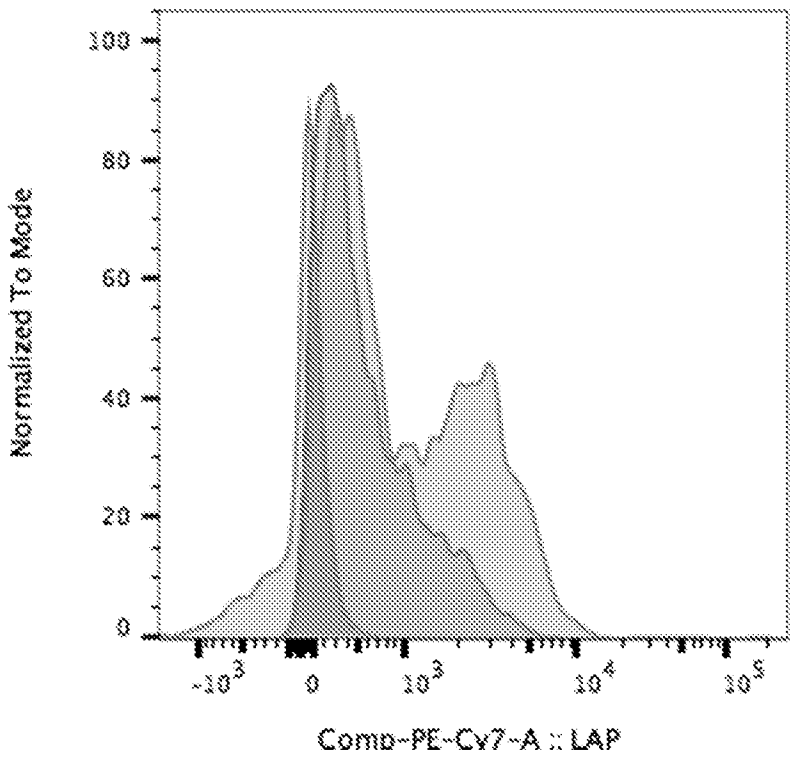
Normalized To Mode
100
80
60
40
20
0
-10^3
0
10^3
10^4
10^5
Comp-PE-Cy7-A :: LAP

FIG. 7A

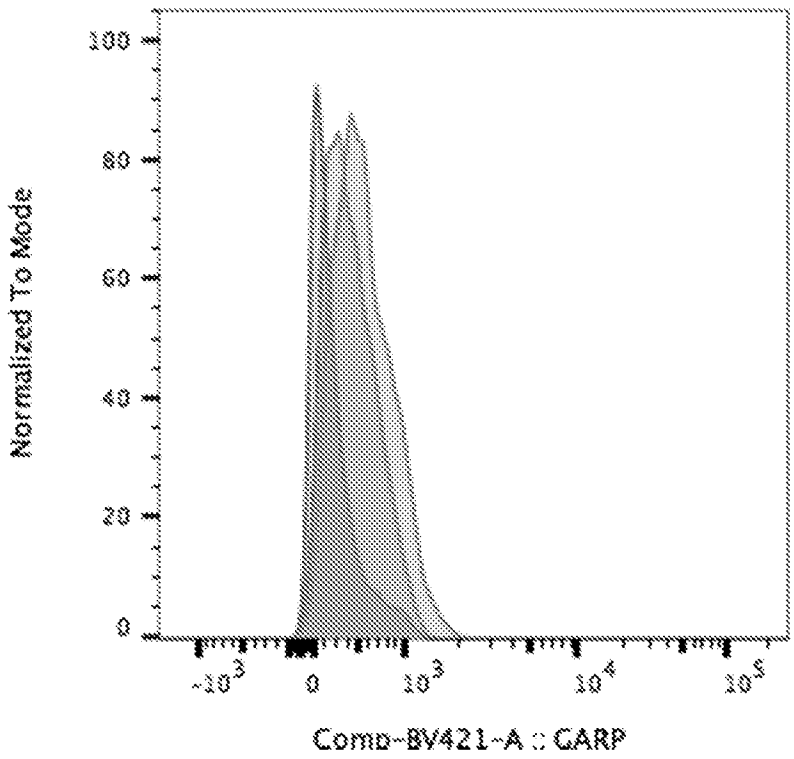

FIG. 7B

FIG. 8A

FIG. 8B

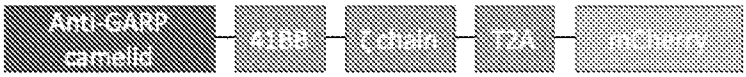

FIG. 8C

FIG. 8D

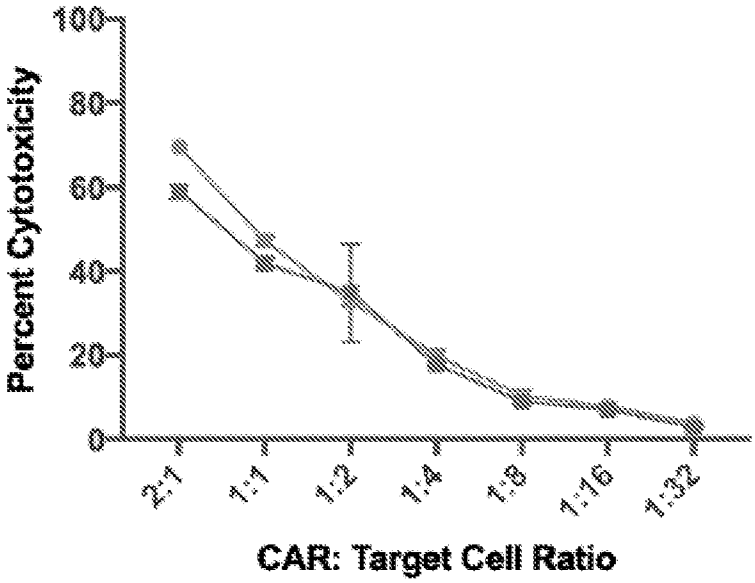
Percent Cytotoxicity
100
80
60
40
20
0
2:1
1:1
1:2
1:4
1:8
1:16
1:32
CAR: Target Cell Ratio

FIG. 9A

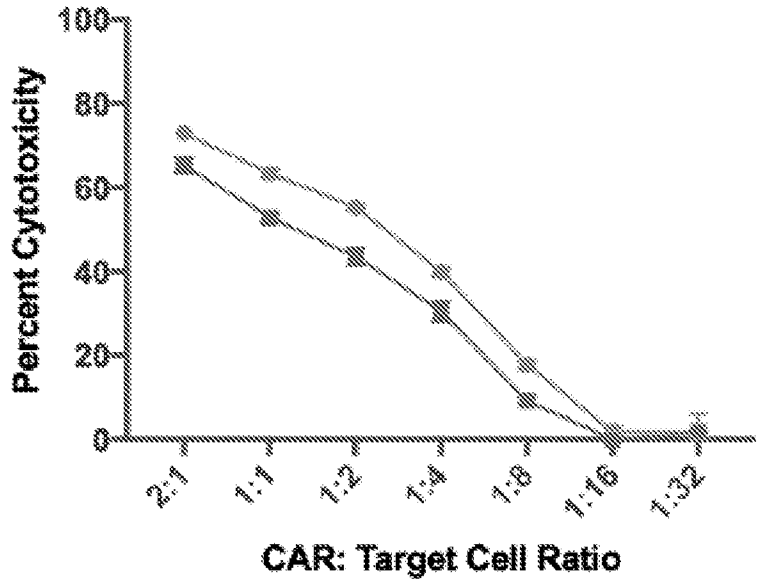
Percent Cytotoxicity
100
80
60
40
20
0
2:1
1:1
1:2
1:4
1:8
1:16
1:32
CAR: Target Cell Ratio

FIG. 9B

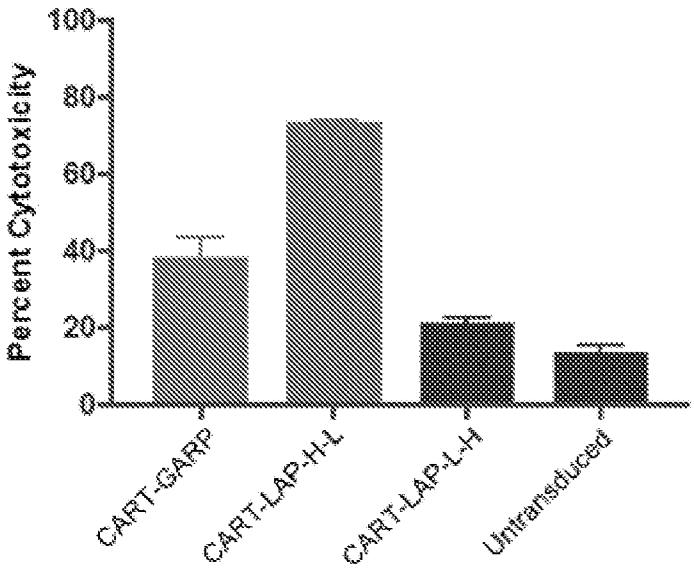

FIG. 10A

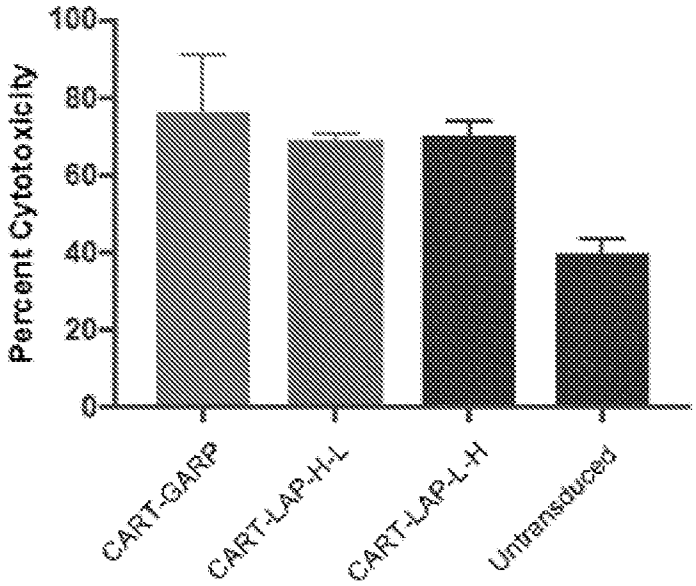

FIG. 10B

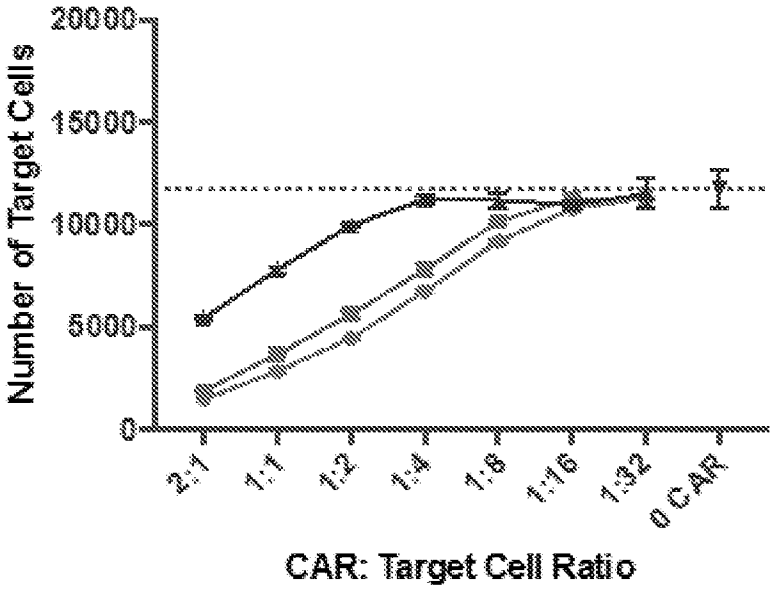

FIG. 11A

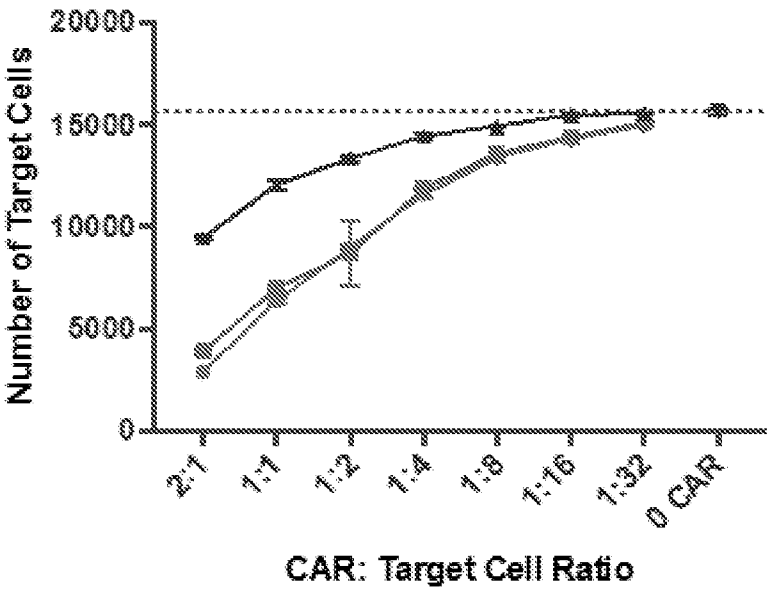

FIG. 11B

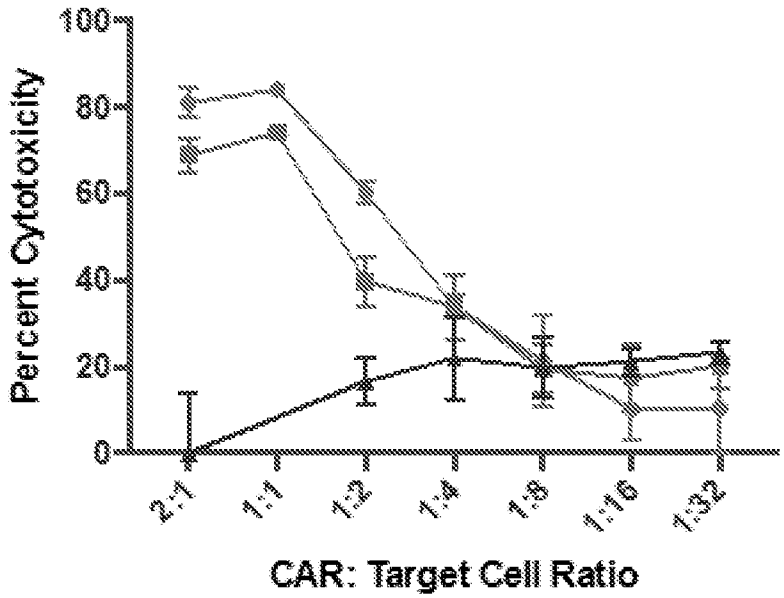

FIG. 11C

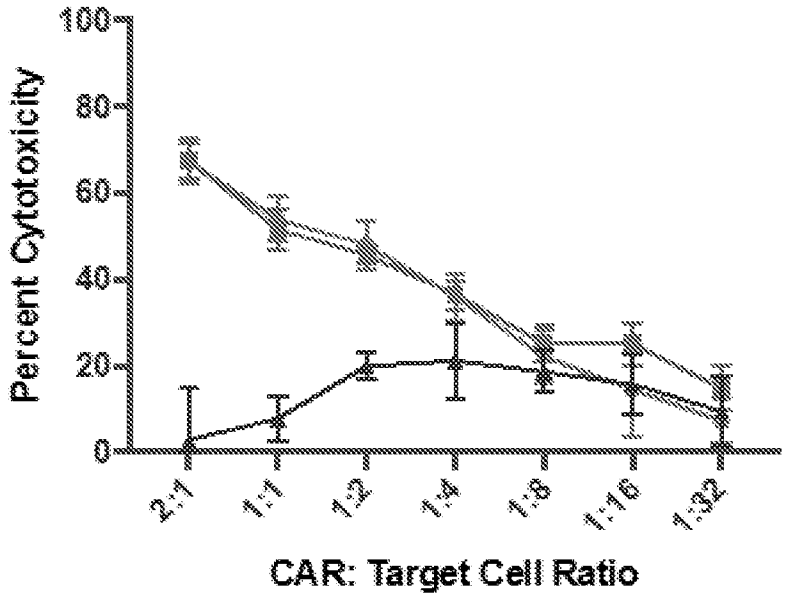

FIG. 11D

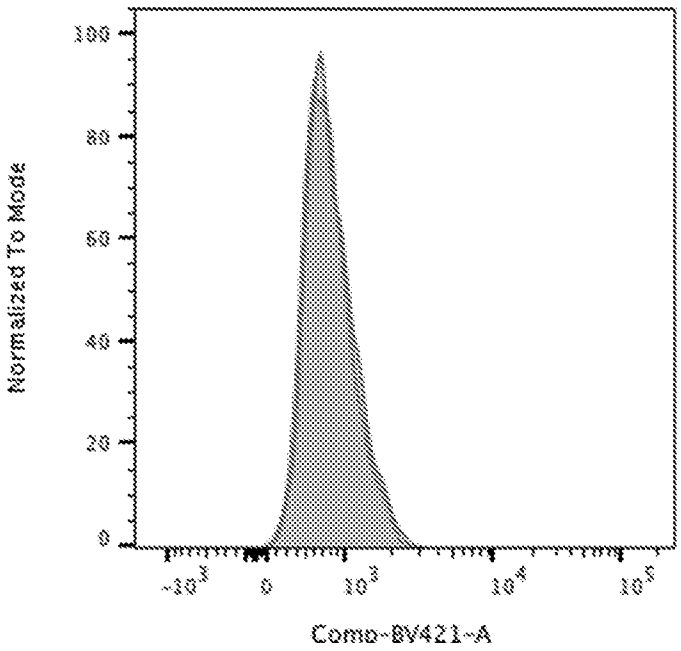

FIG. 12A

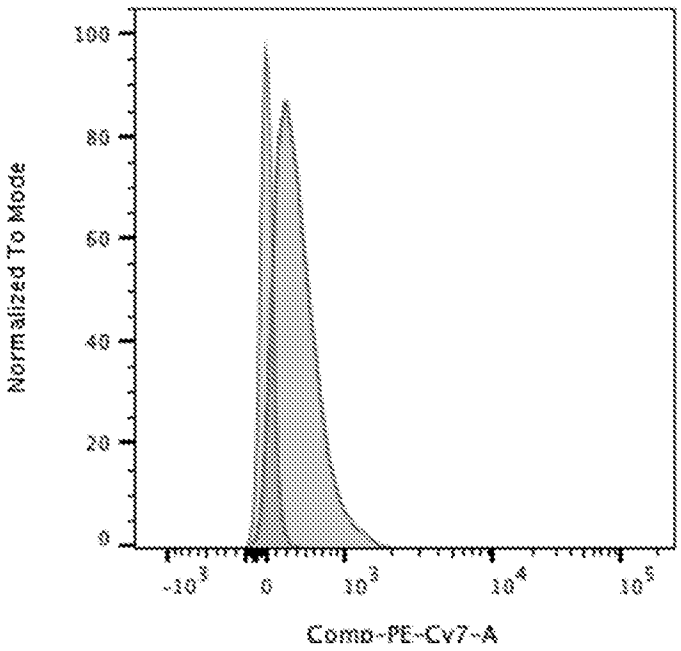

FIG. 12B

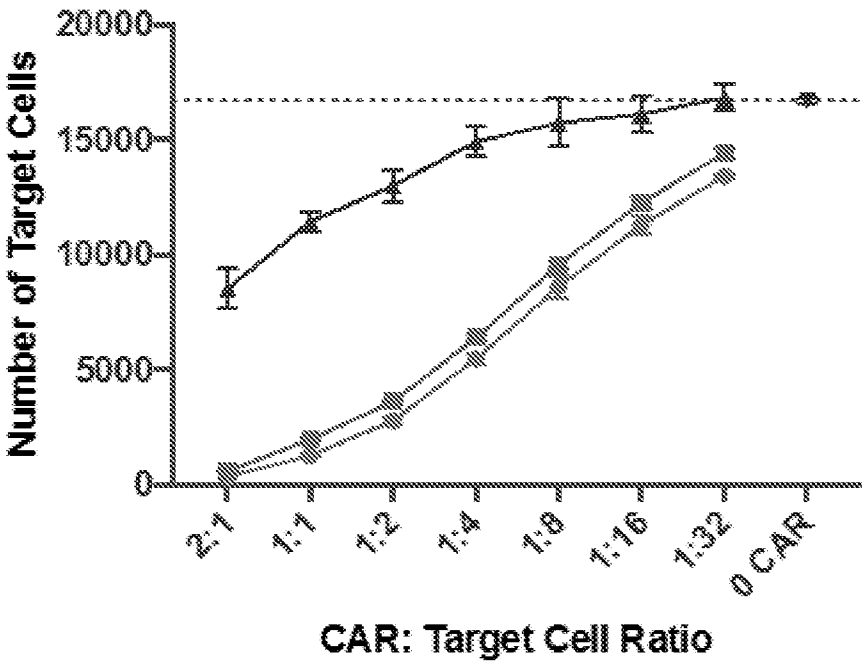

FIG. 13A

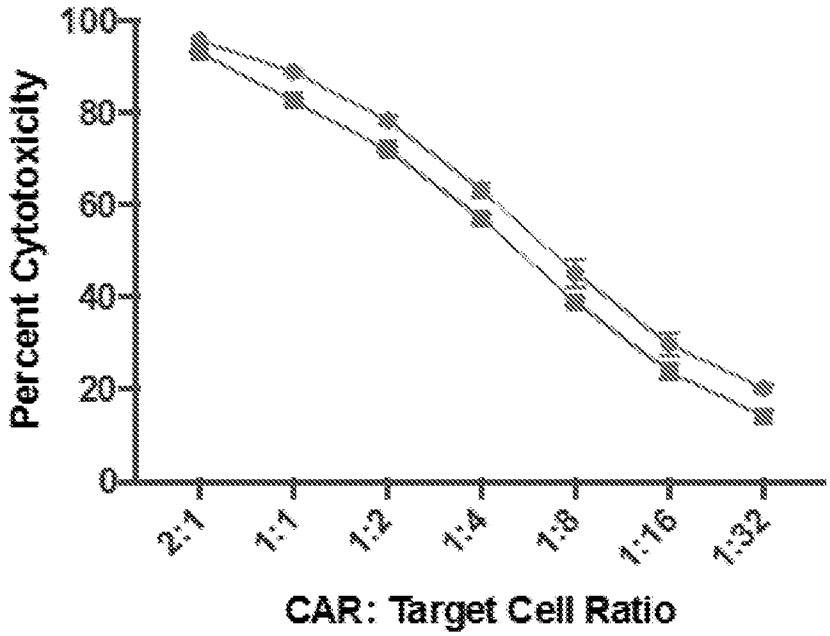

FIG. 13B

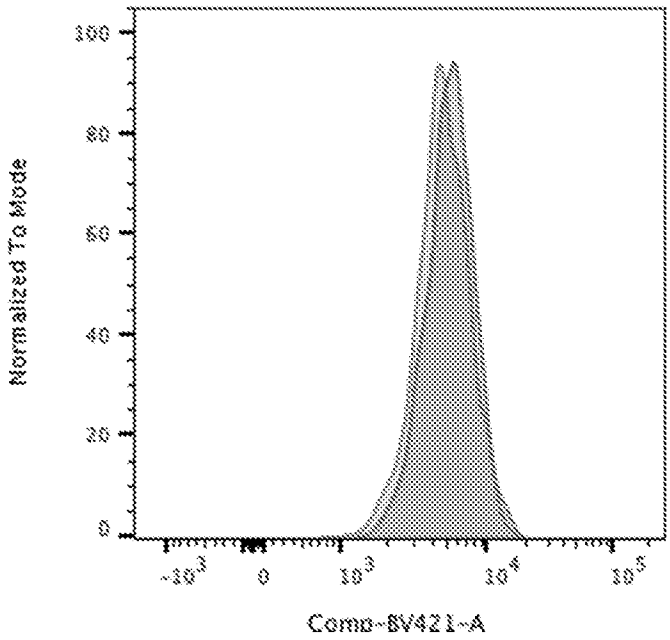

FIG. 14A

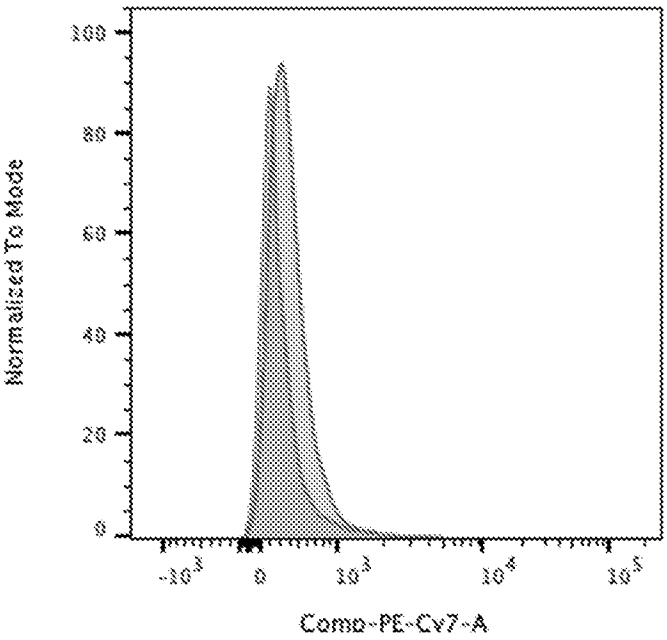

FIG. 14B

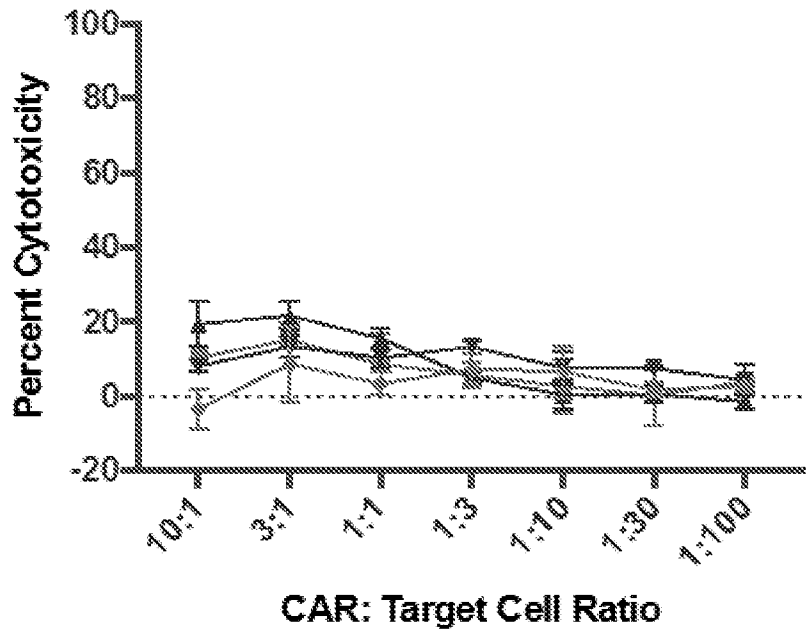

FIG. 15A

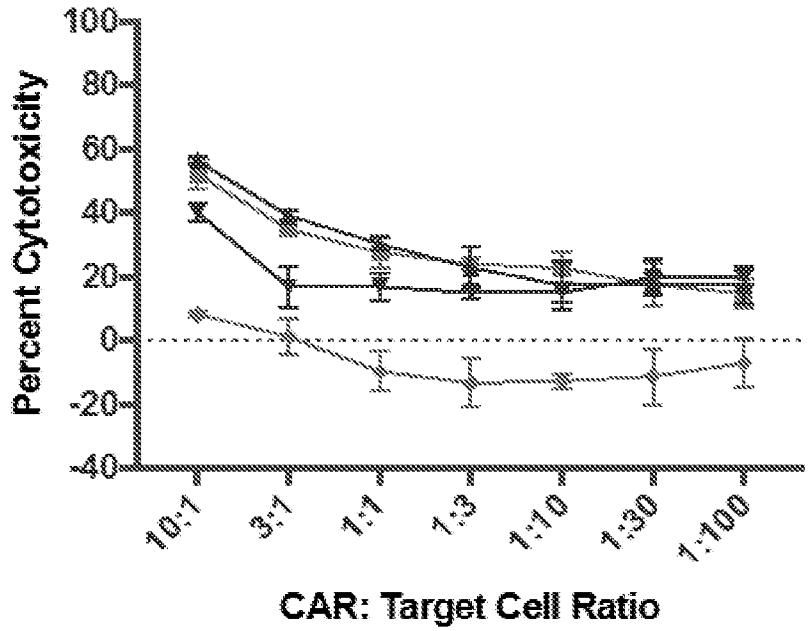

FIG. 15B

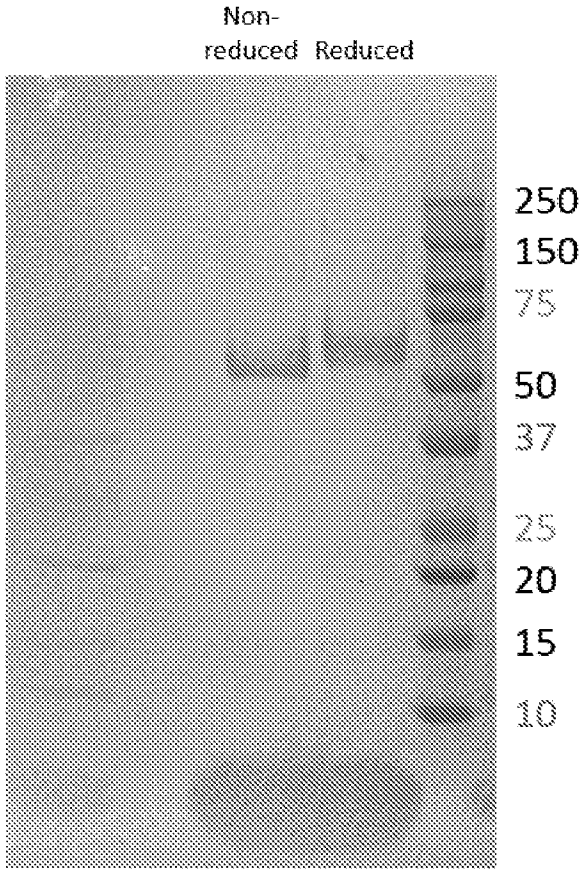

FIG. 16A

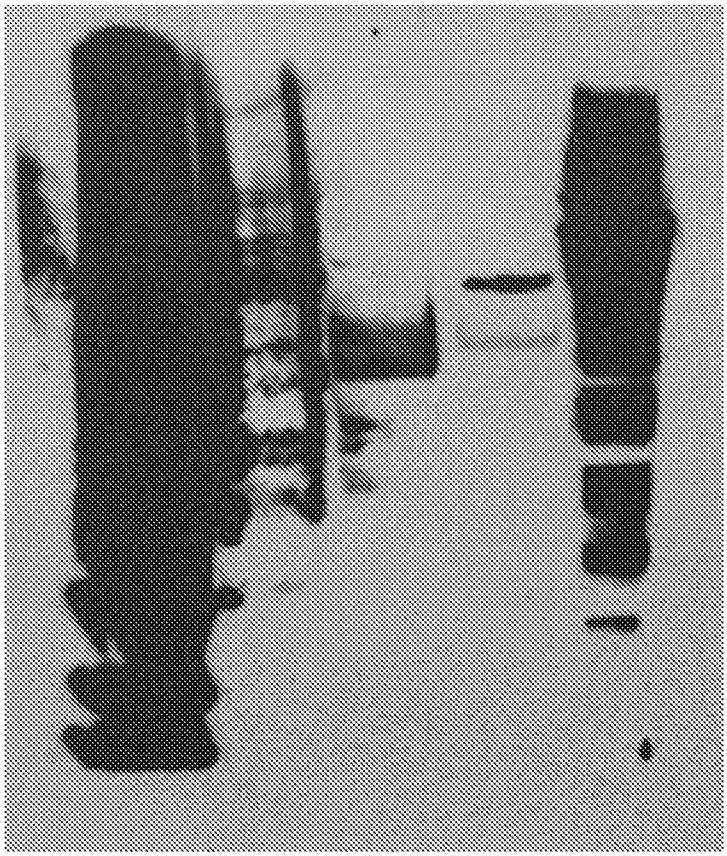

FIG. 16B

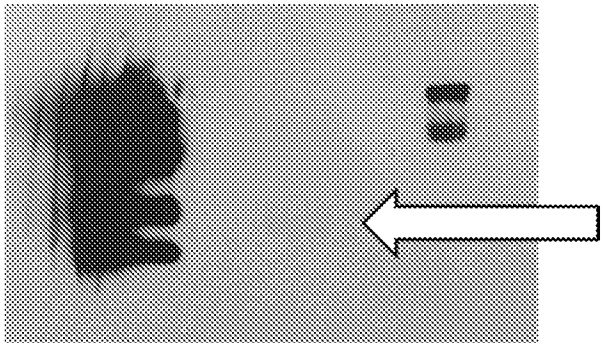

FIG. 16C

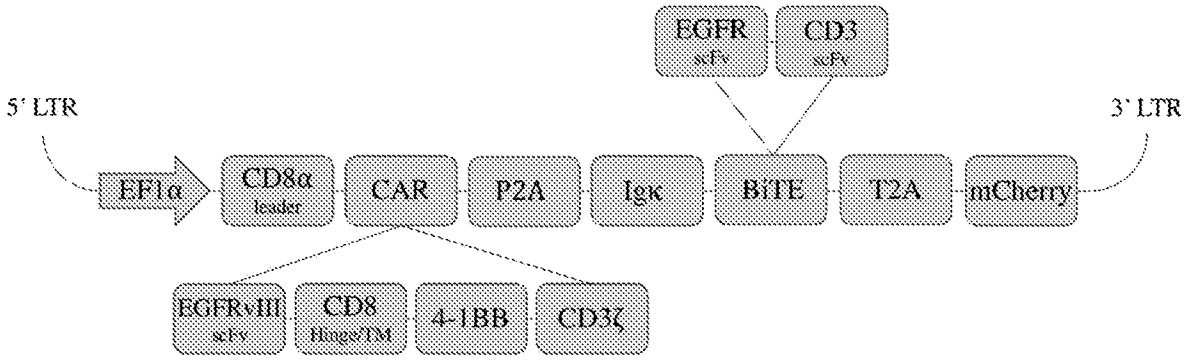

FIG. 17

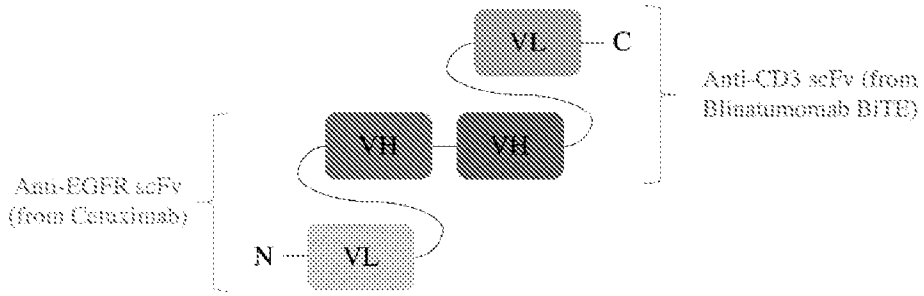

FIG. 18

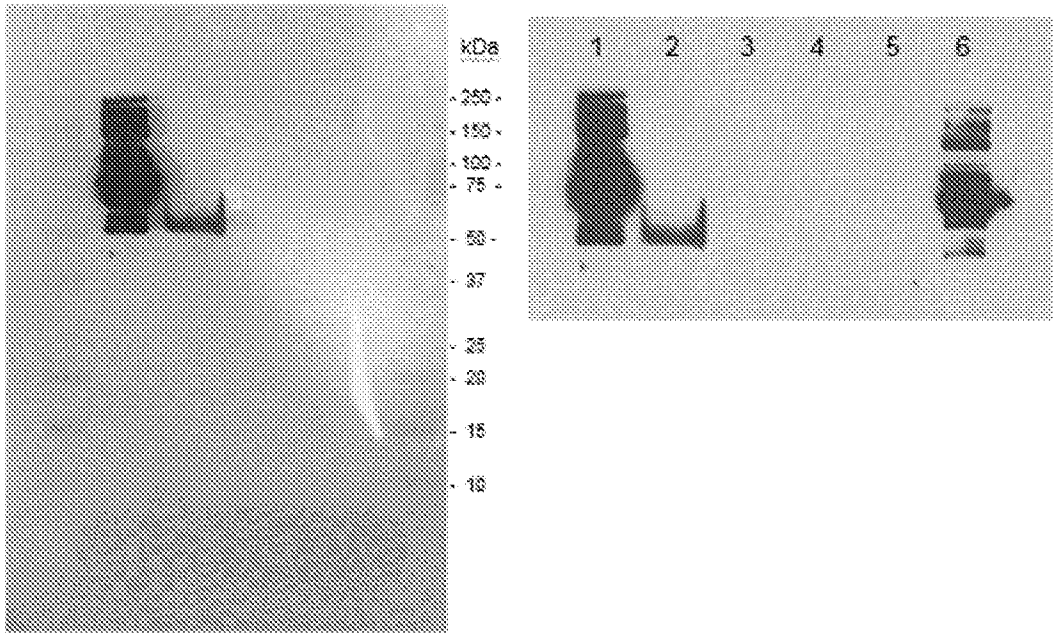

FIG. 19

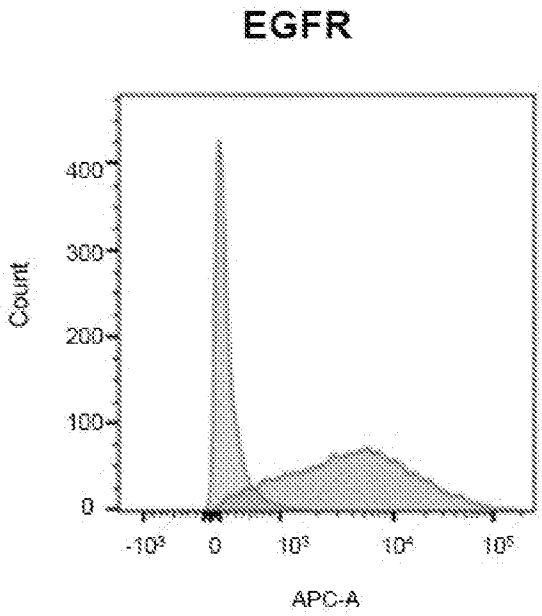

FIG. 20A

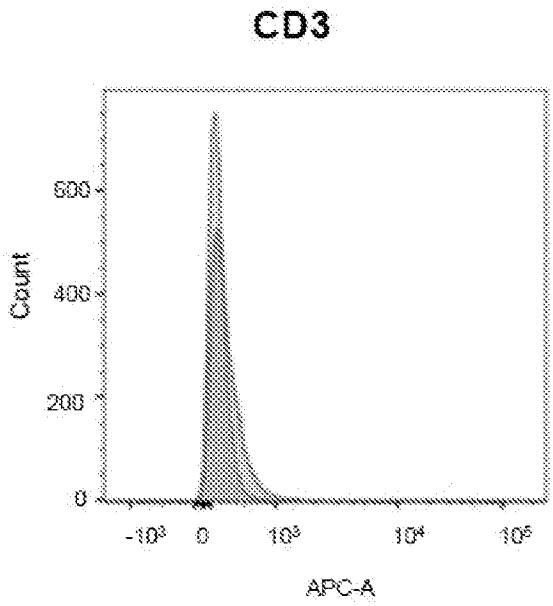

FIG. 20B

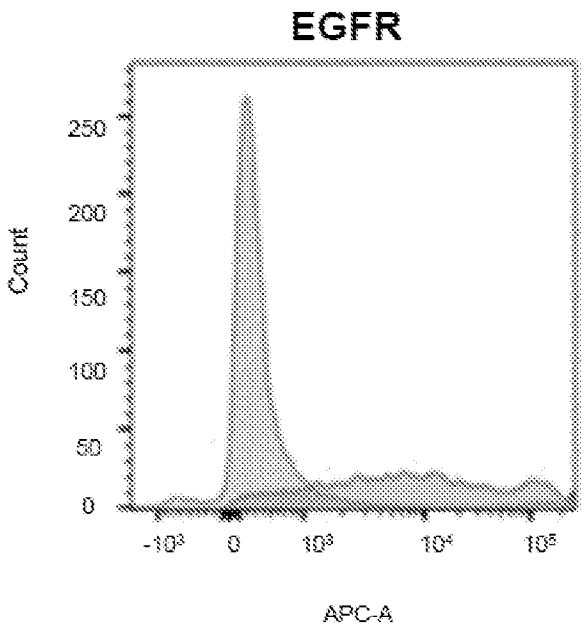

FIG. 21A

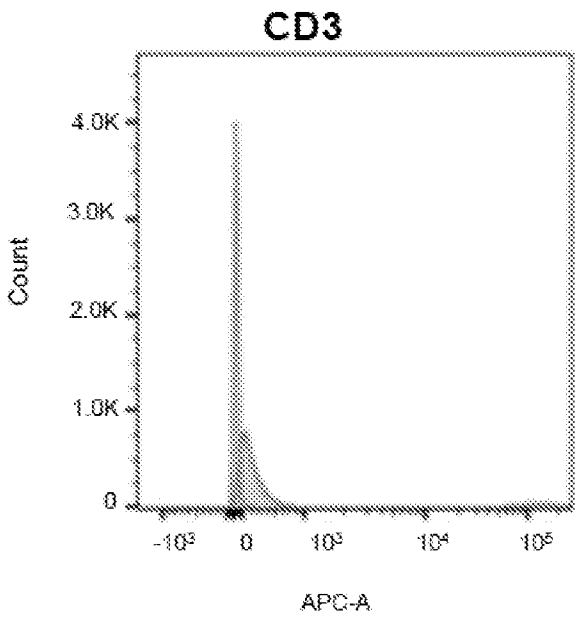

FIG. 21B

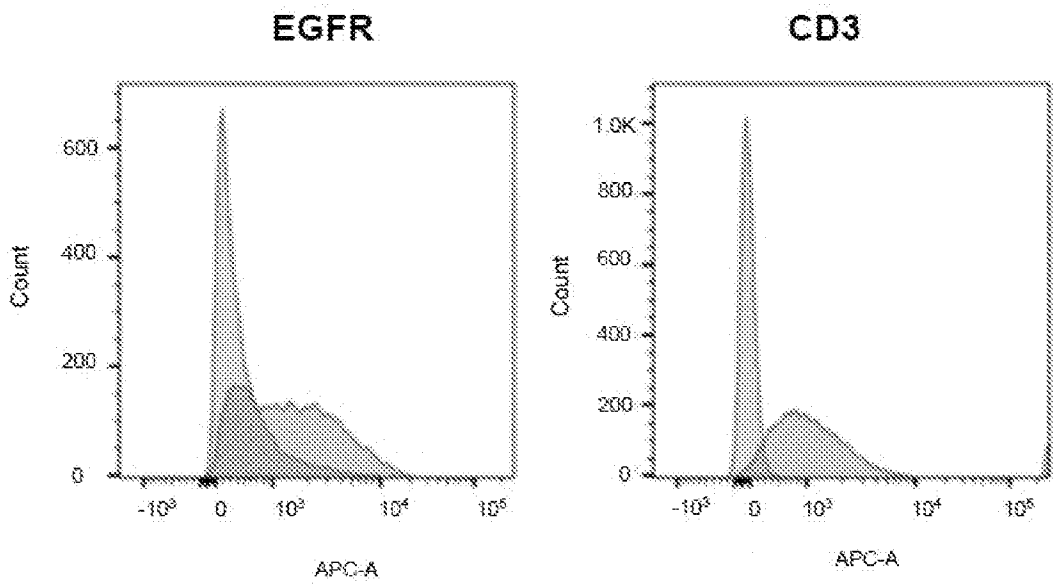

FIG. 22A

FIG. 22B

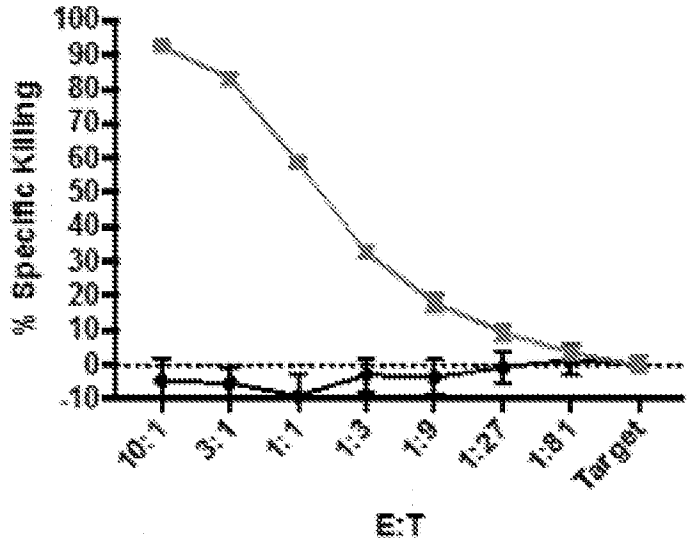

FIG. 23

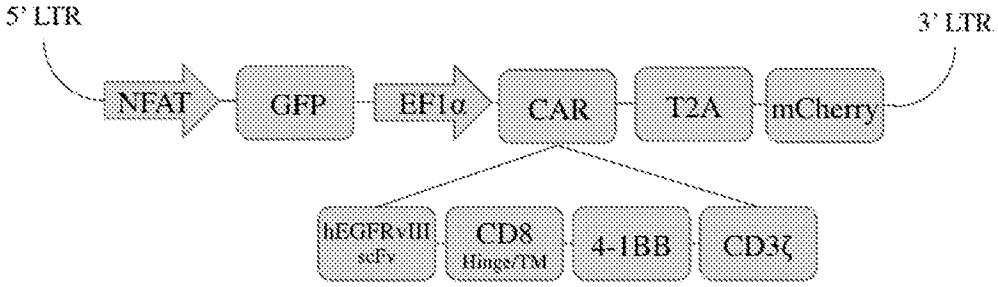

FIG. 24

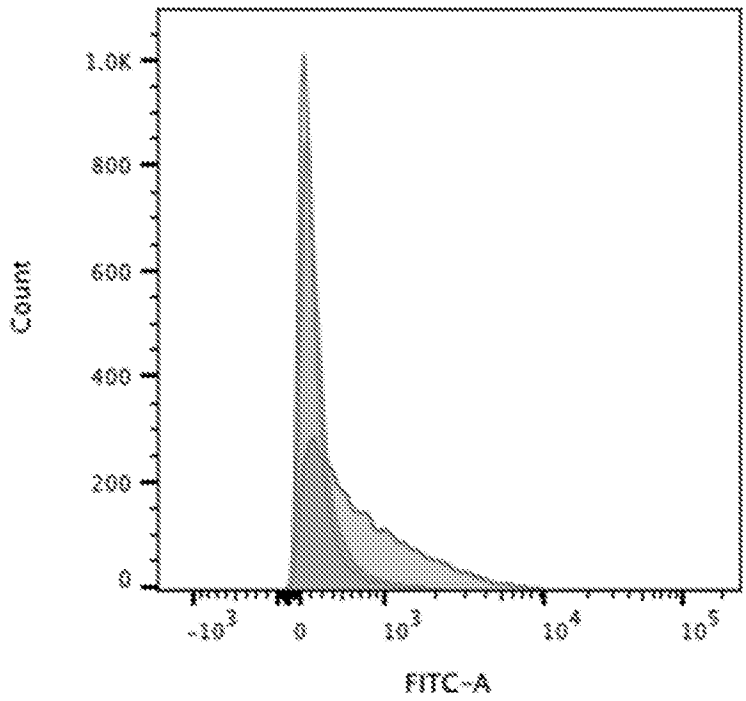

FIG. 25A

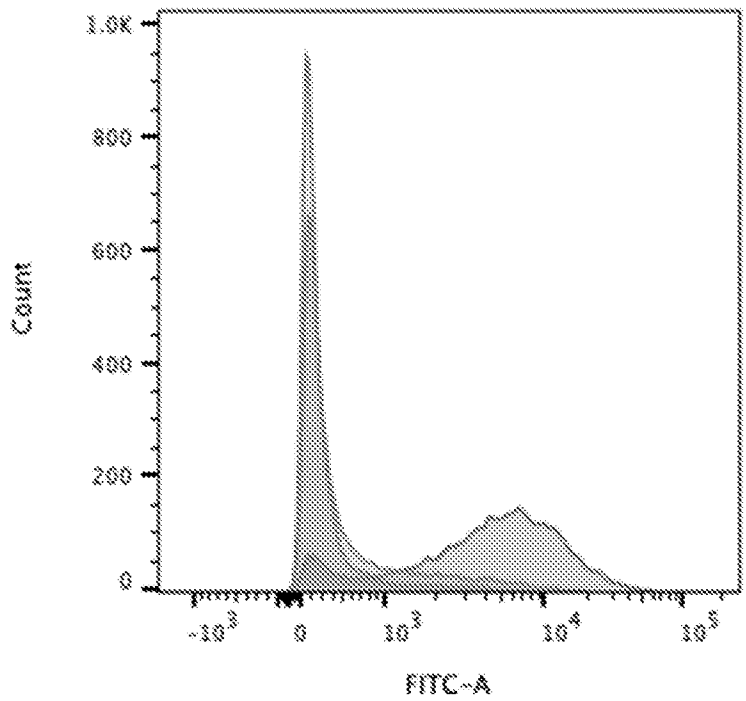

FIG. 25B

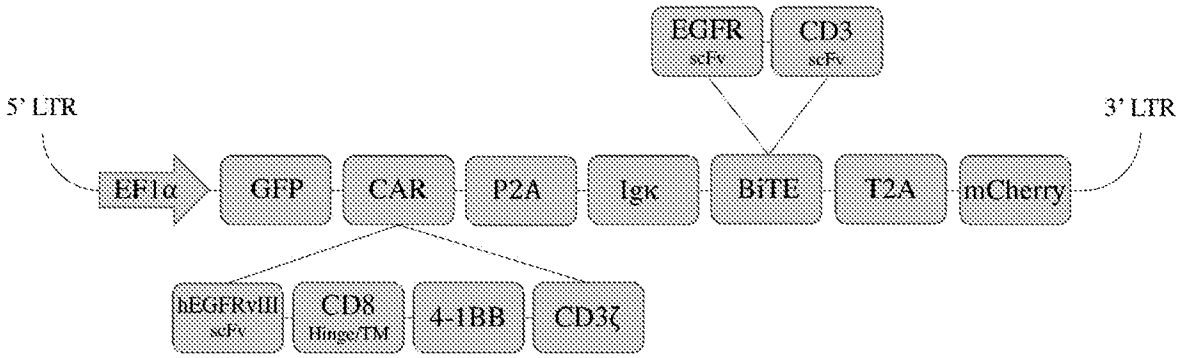

FIG. 26A

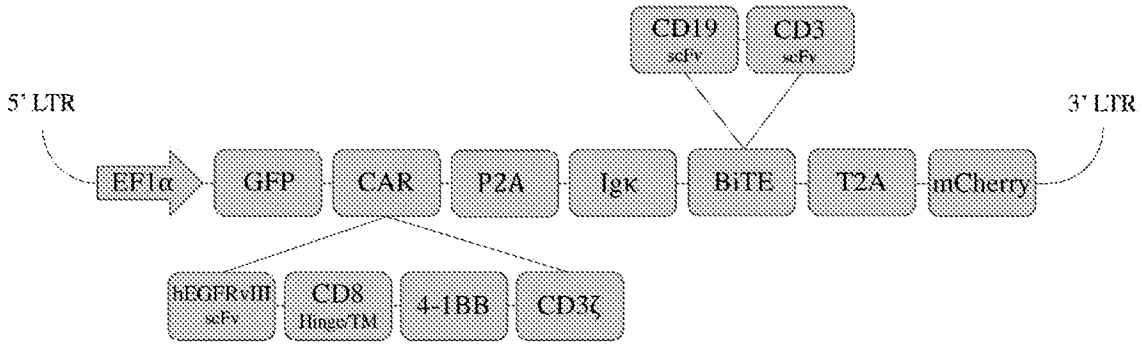

FIG. 26B

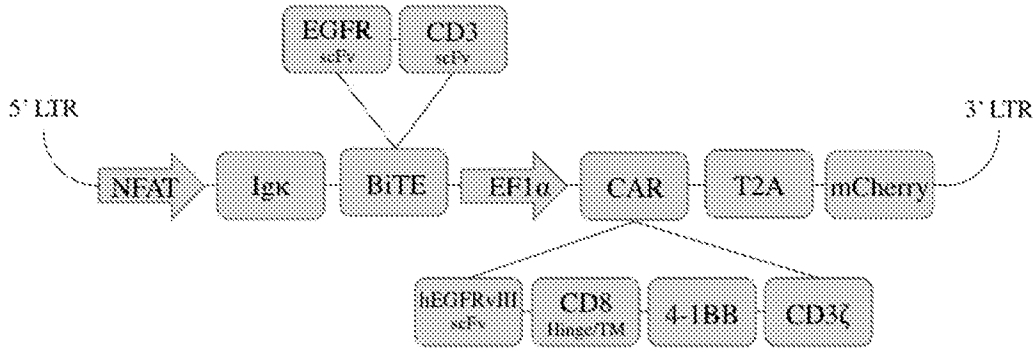

FIG. 27A

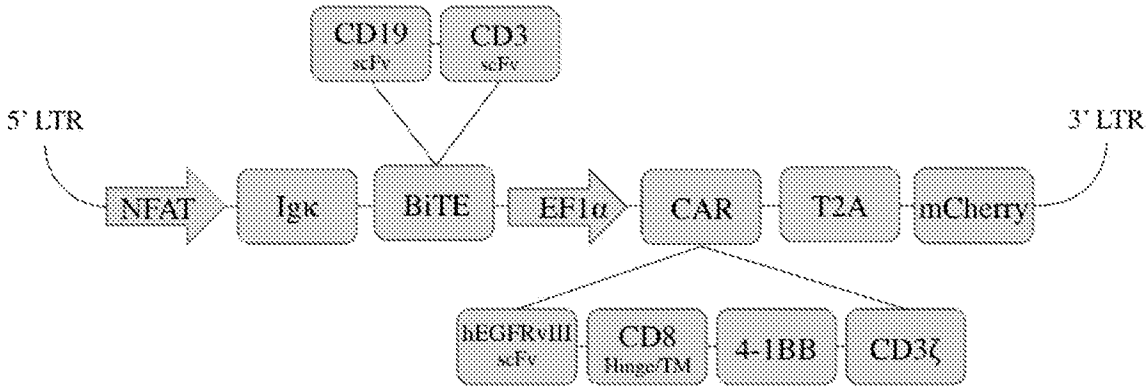

FIG. 27B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27783

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 14/47, 14/52, 14/725, 14/705, 16/18, 16/46, 16/28, 16/30; A61K 35/12, 35/15 (2018.01)
CPC - C07K 14/47, 14/52, 14/705, 14/70503, 16/18, 16/28, 16/30, 16/46, 16/468, 16/2803, 16/2809, 16/2863, 16/2896,16/3053; A61K 35/12, 35/15, 35/17, 38/17, 38/179; C12N 5/0645

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2017/ 049166 A1 (NOVARTIS AG et al.) 23 March 2017; page 7, lines 28-29; page 10, line 21; page 14, line 30; page 22, line 23; page 28, lines 5-6; page 30, lines 10-11; page 72, line 21; page 138, lines 19-21, 23-25; page 155, lines 19-20; page 174, lines 11-12; page 186, line 24; page 189, line 23; page 191, lines 5-6; page 198, line 31; page 206, lines 20-21; page 217, line 19, 25; page 225, line 29; page 238, line 26; page 248, lines 18-20; page 282, lines 20-21; page 292, lines 19-22; page 297, line 30; page 298, lines 5-7; page 314, lines 11-12	1-3, 8-13, 17-25, 33-41 ---------- 26, 42
X	US 2014/0322275 A1 (BROGDON, J et al.) 30 October 2014; paragraphs [0009], [0109], [0115], [0133]-[0134], [0136], [0145], [0210], [0215]	1, 22, 24
Y	US 2016/0272717 A1 (LUDWIG INSTITUTE FOR CANCER RESEARCH LTD et al.) 22 September 2016; paragraphs [0017], [0196], [0219]	26, 42
A	US 9,567,399 B1 (KYMAB LIMITED) 14 February 2017; entire document	1-3, 8-13, 17-26, 33-42
A	US 2014/0322183 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 30 October 2014; entire document	1-3, 8-13, 17-26, 33-42
A	US 2016/0200819 A1 (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERV) 14 July 2016; entire document	1-3, 8-13, 17-26, 33-42

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 2018 (07.08.2018)	24 AUG 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27783

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	(OROURKE, DM et al) A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Science Translation Medicine. 19 July 2019, Vol. 9, No. 399, pages 1-30; DOI: 10.1126/scitranslmed.aaa0984	1-3, 8-13, 17-26, 33-42
E, X	US 2018/0162939 A1 (ICELL GENE THERAPEUTICS LLC) 14 June 2018; entire document	1-3, 8-13, 17-26, 33-42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/27783

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

-Please See Supplemental Page--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 8-13, 17-26, 33-42; SEQ ID NOs: 3, 27

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

-***-Continued from Box No. III: Observations where unity of invention is lacking-***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-43, a single chain antibody (SEQ ID NO: 3) that binds to glycoprotein A repetitions predominant (GARP) (therapeutic agent), a P2A peptide cleavable moiety (cleavable moiety), an EF1a promoter (promoter), and a 3C10 scFv (SEQ ID NO: 27) that binds to EGFRvIII (antigen-binding domain) are directed toward a chimeric antigen receptor (CAR) T cell; a nucleic acid molecule associated therewith; a CAR polypeptide associated therewith; a pharmaceutical composition comprising the CAR T cell; and a method of treating a patient having cancer comprising administering the CAR T cell to the patient.

The CAR T cells, nucleic acid, polypeptide, pharmaceutical composition and methods will be searched to the extent they encompass a therapeutic agent encompassing a single chain antibody encompassing SEQ ID NO: 3 that binds to glycoprotein A repetitions predominant (GARP) (first exemplary therapeutic agent), a P2A peptide cleavable moiety (first exemplary cleavable moiety), an EF1a promoter (first exemplary promoter), and 3C10 scFv, encompassing SEQ ID NO: 27, that binds to EGFRvIII (antigen-binding domain). Applicant is invited to elect additional domain(s) (e.g. therapeutic agent domain, antigen-binding domain), and, where applicable, construct(s) comprising the assembled domain(s), and/or cleavable moiety(ies) and/or promoter(s), with, where applicable, specified SEQ ID NO: for each, such that the sequence of each elected species is fully specified (i.e. no optional or variable residues or substituents), to be searched. Additional specified domain(s) and, where applicable, construct(s) , and/or cleavable moiety(ies) and/or promoter(s) will be searched upon the payment of additional fees. It is believed that claims 1, 2, 3 (in-part), 8-10, 11 (in-part), 12, 13, 17, 18 (in-part), 19-23, 24 (in-part), 25, 26 (in-part), 33-41, and 42 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass a single chain antibody (SEQ ID NO: 3) that binds to glycoprotein A repetitions predominant (GARP) (therapeutic agent), a P2A peptide cleavable moiety (cleavable moiety), an EF1a promoter (promoter), and a 3C10 scFv (SEQ ID NO: 27) that binds to EGFRvIII (antigen-binding domain). Applicants must specify the claims that encompass any additionally elected additional domain(s) (e.g. therapeutic agent domain, antigen-binding domain), and, where applicable, construct (s) comprising the assembled domain(s), and/or cleavable moiety(ies) and/or promoter(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a single domain antibody that binds to LAP (therapeutic agent). (It is noted that the sequences of the CD8 hinge/transmembrane regions, CD3zeta intracellular signaling domains and 4-1BB co-stimulatory domains, despite being indicated to have different SEQ ID NOs, each represent the same, single amino acid sequence for each domain, respectively. Thus the amino acid sequences of each of these domains, represented by the first instance of a SEQ ID NO: for each, will be searched as a part of the first embodiment of Groups I+).

Group II, Claims 44-49 are directed toward a method of delivering a therapeutic antibody, toxin or agent to a tissue or organ in a patient to treat a disease or pathology; wherein the therapeutic antibody, toxin, or agent would, by itself, be unable to enter or penetrate the tissue or organ.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I+ include SEQ ID NO: 4, not present in Group II; the special technical features of Group II include wherein a therapeutic antibody, toxin, or agent would, by itself, be unable to enter or penetrate a tissue or organ, not present in any of Groups I+.

Groups I+ and II share the technical features including: a method comprising delivering or administering a therapeutic agent to a tissue or organ in a patient to treat a disease or pathology, the method comprising administering to said patient a CAR T cell, genetically modified to secrete a therapeutic antibody, toxin, or agent. However, these shared technical features are previously disclosed by US 2017/0008963 A1 to Brogdon et al. (hereinafter 'Brogdon').

rogdon discloses a method comprising delivering or administering a therapeutic agent to a tissue or organ in a patient to treat a disease or pathology (a method comprising delivering or administering a cell that produces a PD1 inhibitor (a therapeutic agent) to a tissue or organ in a patient to treat a disease or pathology paragraphs [0035], [0332]), the method comprising administering to said patient a CAR T cell, genetically modified to secrete a therapeutic antibody, or agent (the method comprising administering to said patient a CAR T cell, genetically modified to secrete a PD1 inhibitor (therapeutic antibody, or agent); paragraphs [0035], [0332]).

No technical features are shared between the antigen-binding domains and/or therapeutic agents and/or promoters and/or cleavable domains of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a chimeric antigen receptor (CAR) T cell comprising a heterologous nucleic acid molecule, wherein the heterologous nucleic acid molecule comprises: (a) a first polynucleotide encoding a CAR comprising an antigen-binding domain, a CD8 hinge/transmembrane domain comprising SEQ ID NO: 4, a 4-1BB co-stimulatory domain, which comprises SEQ ID NO: 5 and an intracellular signaling domain comprising a CD3 zeta domain, comprising SEQ ID NO: 6; and (b) a second polynucleotide encoding a therapeutic agent; a nucleic acid molecule encoding (i) a CAR polypeptide and a therapeutic agent; a CAR polypeptide, or polyprotein comprising a CAR polypeptide and a therapeutic agent; a pharmaceutical composition comprising one or more CAR T cells, nucleic acid molecules, CAR polypeptides, or a polyprotein; a method of treating a patient having cancer, the method comprising administering to the patient a pharmaceutical composition comprising one or more CAR T cell or a nucleic acid molecule, CAR polypeptide, or polyprotein thereof; and a method of treating a patient having cancer, the method comprising administering to the patient a CAR T cell product, genetically modified to secrete a tumor-toxic antibody, wherein by directing the cancer toxicity locally to the tumor microenvironment, systemic toxicity is reduced; these shared technical features are previously disclosed by Brogdon, as above, in view of US 2016/0272717 A1 to Ludwig Institute for Cancer Research, Ltd. et al. (hereinafter 'Ludwig').

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Previous Supplemental Page:

Brogdon discloses a chimeric antigen receptor (CAR) T cell (a chimeric antigen receptor (CAR) T cell; abstract, paragraphs [0007], [0031]) comprising a heterologous nucleic acid molecule (comprising a heterologous nucleic acid molecule; paragraphs [0008], [0031]), wherein the heterologous nucleic acid molecule comprises: (a) a first polynucleotide encoding a CAR (wherein the heterologous nucleic acid molecule comprises: (a) a first polynucleotide encoding a CAR; paragraphs [0008]) comprising an antigen-binding domain (comprising a 3C10 anti-EGFRvIII binding domain (an antigen-binding domain), comprising SEQ ID NO: 11 (SEQ ID NO: 27); paragraphs [0009], [0136], SEQ ID NO: 11; wherein SEQ ID NO: 11 is 100% identical to Applicants' SEQ ID NO: 27), a CD8 hinge/transmembrane domain comprising SEQ ID NO: 4 (a CD8 hinge/transmembrane domain, comprising residues 268-336 of SEQ ID NO: 43; paragraphs [0015], [0195], wherein residues 268-336 of SEQ ID NO: 43 are 100% identical to Applicants' SEQ ID NO: 4), a 4-1BB co-stimulatory domain, which comprises SEQ ID NO: 5 (a 4-1BB costimulatory domain comprising SEQ ID NO: 16, and residues 337-378 of SEQ ID NO: 43; paragraph [0013], SEQ ID NO: 16 and residues 337-378 of SEQ ID NO: 43; wherein SEQ ID NO: 16 and residues 337-378 of SEQ ID NO: 43 are 100% identical to Applicants' SEQ ID NO: 5) and an intracellular signaling domain comprising a CD3 zeta domain, comprising SEQ ID NO: 6 (and an intracellular signaling domain comprising a CD3 zeta domain, comprising SEQ ID NO: 17, and residues 379-490 of SEQ ID NO: 43 (SEQ ID NO: 6); paragraphs [0013], SEQ ID NO: 17 and residues 379-490 of SEQ ID NO: 43; wherein SEQ ID NO: 17 and residues 379-490 of SEQ ID NO: 43 are 100% identical to Applicants' SEQ ID NO: 6); and (b) a second polynucleotide encoding a therapeutic agent (a nucleic acid encoding an inhibitor of an inihibitory molecule, linked to the nucleic acid that encodes the CAR (a second polynucleotide encoding a therapeutic agent); paragraphs [0331], [0332]); a nucleic acid molecule encoding (i) a CAR polypeptide and a therapeutic agent (a nucleic acid molecule encoding (i) a CAR polypeptide and a therapeutic agent; paragraphs [0331], [0332]); a CAR polypeptide, or polyprotein comprising a CAR polypeptide and a therapeutic agent (a CAR polypeptide, or polyprotein comprising a CAR polypeptide and a therapeutic agent; paragraph [0332]); a pharmaceutical composition comprising one or more CAR T cells, nucleic acid molecules, CAR polypeptides, or a polyprotein (a pharmaceutical composition comprising one or more CAR T cells, nucleic acid molecules, CAR polypeptides, or a polyprotein; paragraph [0312]); a method of treating a patient having cancer (a method of treating a patient having cancer; paragraph [0035]), the method comprising administering to the patient a pharmaceutical composition comprising one or more CAR T cell or a nucleic acid molecule, CAR polypeptide, or polyprotein thereof (the method comprising administering to the patient a pharmaceutical composition comprising one or more CAR T cell or a nucleic acid molecule, CAR polypeptide, or polyprotein thereof; paragraphs [0035], [0312], [0331], [0332]); and a method of treating a patient having cancer (a method of treating a patient having cancer; paragraph [0035]), the method comprising administering to the patient a CAR T cell product (the method comprising administering to the patient a CAR T cell pharmaceutical composition (product); paragraphs [0035], [0312]), genetically modified to secrete a tumor-toxic antibody (genetically modified to express on the surface (secrete) a tumor-targeted CAR with an antibody extracellular domain that causes specific killing of tumor cells (a tumor-toxic antibody); paragraphs [0008], [0050], [0060]).

Brogdon does not disclose: a second polynucleotide encoding a therapeutic agent comprising a single chain antibody (SEQ ID NO: 3) that binds to glycoprotein A repetitions predominant (GARP), wherein the single chain antibody comprises SEQ ID NO: 3; and wherein by directing the cancer toxicity locally to the tumor microenvironment, systemic toxicity is reduced.

Ludwig discloses an antibody that binds to glycoprotein A repetitions predominant (GARP) (an antibody that binds to glycoprotein A repetitions predominant (GARP); abstract), including a single chain antibody (a single chain antibody; paragraph [0013]) comprising SEQ ID NO: 3 (comprising a light chain variable region encompassing SEQ ID NO: 39 (SEQ ID NO: 3); paragraph [0040], wherein SEQ ID NO: 39 is 100% identical to Applicants' SEQ ID NO: 3), when the GARP is complexed with TGF-B produced by immunosuppresiv T reg cells (when the GARP is complexed with TGF-B produced by immunosuppresive T reg cells; paragraphs [0005], [0006], [0012]); and treatment of cancer therewith (and treatment of cancer therewith; paragraphs [0001], [0047]).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Brogdon to have described the specific killing of tumor cells by the modified T-cells disclosed by Brogdon as directing the cancer toxicity locally to the tumor microenvironment, and reducing systemic toxicity associated with the administration of a second therapeutic agent, such as an inhibitor of immune inhibitory molecules, as disclosed by Brogdon, based on the specific killing of the desired tumor cells, as disclosed by Brogdon, and by the production of the second therapeutic as a fusion molecule with the heterologous CAR by the modified T cells, which bind preferentially to the target tumor cells. It further would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Brogdon to have used a secondary agent comprising an antibody domain that binds to a target produce by immunosuppressive T reg cells, such as TGF-B complexed with GARP, as disclosed by Ludwig, for the effective treatment of cancer by specifically targeting and eliminating or inhibiting immunosuppressive T reg cells in the tumor, based on tumor targeting of the modified T cells producing a CAR, as disclosed by Brogdon, as well as a GARP-TGFB binding moiety, as disclosed by Ludwig, in order to enhance the immune response against the tumor to provide more effective treatment for cancer.

Since none of the special technical features of the Groups I+ and II inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Brogdon and Ludwig references, unity of invention is lacking.