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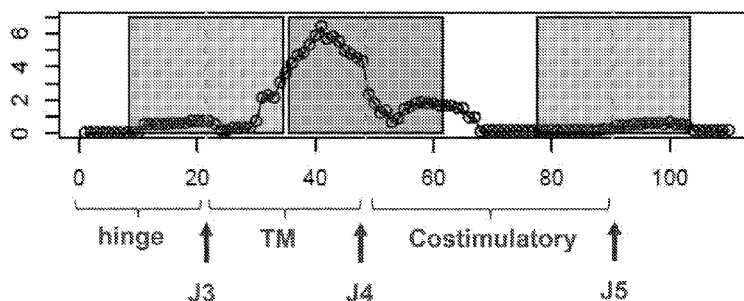
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(54) Title: MODIFIED CHIMERIC RECEPTORS AND RELATED COMPOSITIONS AND METHODS

FIG. 4A



(57) Abstract: Provided are chimeric receptors for engineering cells for adoptive therapy, including T cells, and the genetically engineered cells. In some embodiments, the chimeric receptors, such as chimeric antigen receptors (CARs) are modified in a junction region by one or more amino acid modifications such that peptide fragments of such region exhibit a lower binding affinity for a human leukocyte antigen (HLA) and/or the region exhibits reduced immunogenicity, including following administration to a subject. In some aspects, also provided are methods and compositions for engineering and producing cells expressing such chimeric receptors, compositions containing the cells, and method for their administration to subjects. In some embodiments, features of the chimeric receptors and engineered cells containing the chimeric receptors result in methods that provide for increased or improved activity, efficacy and/or persistence.

MODIFIED CHIMERIC RECEPTORS AND RELATED COMPOSITIONS AND METHODS

Cross-Reference to Related Applications

[0001] This application claims priority from U.S. provisional application No. 62/348,130 filed June 9, 2016, entitled “Modified Chimeric Receptors and Related Compositions and Methods,” and U.S. provisional application No. 62/262,911 filed December 3, 2015, entitled “Modified Chimeric Receptors and Related Compositions and Methods,” the contents of each of which is incorporated by reference in its entirety.

Incorporation by Reference of Sequence Listing

[0002] The present application is being filed with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 735042004740SeqList.txt, created November 30, 2016, which is 71,888 bytes in size. The information in electronic format of the Sequence Listing is incorporated by reference in its entirety.

Field

[0003] The present disclosure relates in some aspects to chimeric receptors for engineering cells for adoptive therapy, including T cells, and the genetically engineered cells. In some embodiments, the chimeric receptors, such as chimeric antigen receptors (CARs) are modified in a junction region by one or more amino acid modifications such that peptide fragments of such region exhibit a lower binding affinity for a human leukocyte antigen (HLA) and/or the region exhibits reduced immunogenicity, including following administration to a subject. In some aspects, the disclosure further relates to methods and compositions for engineering and producing cells expressing such chimeric receptors, compositions containing the cells, and method for their administration to subjects. In some embodiments, features of the chimeric receptors and engineered cells containing the chimeric receptors result in methods that provide for increased or improved activity, efficacy and/or persistence.

Background

[0004] Various methods are available for adoptive cell therapy using engineered cells expressing recombinant receptors, such as chimeric antigen receptor (CARs). Such methods may benefit from enhanced expansion and/or persistence of the administered cells, reduction of the immune response to the receptors expressed on the cells or other unwanted outcomes. Provided are products, compositions, methods and articles of manufacture that meet such needs.

Summary

[0005] Provided herein is a variant chimeric receptor that is modified compared to a reference chimeric receptor by one or more amino acid sequence modifications in a junction region, said junction region being a region containing a contiguous sequence of amino acids on each side of a junction between two domains, i.e. region spanning two domains. In some embodiments, provided herein is a variant chimeric receptor including a modified junction region having one or more amino acid sequence modifications compared to a junction region of the reference chimeric receptor, wherein the reference chimeric receptor includes a first domain and a second domain, joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor includes up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and a peptide fragment having the sequence of an 8-15 amino acid portion of the modified junction region has a binding affinity for a human leukocyte antigen (HLA) molecule that is lower than the binding affinity, for the same HLA molecule, of a peptide fragment having the sequence of the corresponding portion of the junction region of the reference chimeric receptor. In some embodiments, the peptide fragment of the corresponding portion of the junction region of the reference chimeric receptor has a binding affinity of less than 1000 nM, less than 500 nM or less than 50 nM.

[0006] In some embodiments, provided herein is a variant chimeric receptor including a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein the reference chimeric receptor includes a first domain and a second domain, joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor includes up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and the average of the binding affinities of all 8-15 amino acid

fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the modified junction region for a human HLA molecule is lower than the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the junction region of the reference chimeric receptor.

[0007] In some embodiments, the binding affinity or average of binding affinities is more than 2-fold, more than 5-fold, more than 10-fold, more than 25-fold, more than 50-fold or more than 100-fold lower.

[0008] In some embodiments, provided herein is a variant chimeric receptor including a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein the reference chimeric receptor includes a first domain directly linked to a second domain joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor includes up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and the number of peptide fragments having the sequence of an 8-15 amino acid portion of the modified junction region that has a binding affinity for a human leukocyte antigen (HLA) of less than 1000 nM is reduced compared to the number of peptide fragments having the sequence of an 8-15 amino acid portion of the junction region of the reference chimeric receptor that has the same affinity for binding the same HLA.

[0009] In some embodiments, the number of peptide fragments within the modified junction region that exhibits a binding affinity for an HLA of less than 500 nM or less than 50 nM is reduced; or the binding affinity of less than 1000 nM is a binding affinity of less than 500 nM or less than 50 nm. In some embodiments, the binding affinity is an IC₅₀ and the comparison of binding of peptide fragments of the modified junction regions and the peptide fragments of the junction region of the reference chimeric receptor is with reference to the same standard peptide.

[0010] In some embodiments, the first domain and/or second domain of the reference chimeric receptor includes a domain of a natural human protein; and/or the first domain and/or second domain includes an extracellular binding domain, a hinge domain, a transmembrane domain, or an intracellular signaling domain, which intracellular signaling domain is, optionally, a costimulatory signaling domain or an activating cytoplasmic signaling domain. In some embodiments, the first domain and second domain of the reference chimeric receptor are not present in the same molecule *in vivo* in a human subject.

[0011] In some embodiments, the first domain and second domain of the reference chimeric receptor are, respectively, an extracellular ligand binding domain and a hinge domain, a hinge domain and a transmembrane domain, a transmembrane domain and an intracellular costimulatory signaling domain, and an intracellular costimulatory signaling domain and an activating cytoplasmic signaling domain, which can include functional portions of such domains. In some embodiments, the first domain is a transmembrane domain or a functional portion thereof and the second domain is a costimulatory signaling domain or a functional portion thereof.

[0012] In some embodiments, the transmembrane domain is a CD28 transmembrane domain or a functional portion or variant thereof and the costimulatory signaling domain is a 4-1BB signaling domain or a functional portion or variant thereof.

[0013] In some embodiments, the junction region of the reference chimeric receptor includes up to 13 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction. In some embodiments, the peptide fragment(s) includes a sequence of amino acids between or between about 8 and 15 amino acids in length, or includes a sequence of amino acids that is at least or at least about or is or is about 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in length.

[0014] In some embodiments, the variant chimeric receptor includes a domain of at least 95% sequence identity to the first domain and/or a domain of at least 95% sequence identity to the second domain, a domain identical in sequence to the first domain and a domain of at least 95% sequence identity to the second domain, or a domain of at least 95% sequence identity to the first domain and a domain identical in sequence to the second domain, wherein at least one or both of the domains present in the variant chimeric receptor is modified compared to the first domain and/or second domain of the reference chimeric receptor in the portion including the modified junction region.

[0015] In some embodiments, the variant chimeric receptor includes at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the reference chimeric receptor; and/or the variant chimeric receptor includes up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid modifications compared to the reference chimeric receptor.

[0016] In some embodiments, the CD28 transmembrane domain includes the sequence of amino acids set forth in SEQ ID NO:2, 103 or 104 or a functional portion or variant thereof including a sequence that exhibits at least 95% sequence identity to SEQ ID NO:2, 103 or 104; and the 4-1BB costimulatory signaling domain includes the sequence of amino acids set forth in SEQ ID NO:3 or a functional portion or variant thereof including a sequence that exhibits at least 95% sequence identity to SEQ ID NO:3.

[0017] In some embodiments, the first domain and second domain together include the sequence of amino acids set forth in SEQ ID NO:5 or a functional portion or variant thereof including a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:5. In some embodiments, the first domain and second domain together include the sequence of amino acids set forth in SEQ ID NO:5. In some embodiments, the one or more modifications are within a portion between residue 13 and 42 or between amino acid residue 15 and 40, with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the one or more modifications include an amino acid insertion, replacement or deletion and/or wherein each of the one or more modifications individually includes an amino acid insertion, replacement, or deletion.

[0018] In some embodiments, the first and/or second domain of the reference chimeric receptor includes a transmembrane domain, wherein the one or more modifications of the variant chimeric receptor is not or does not include a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, which optionally is the CD28 transmembrane domain; or the one or more modifications include a modification of a hydrophobic amino acid residue within the transmembrane domain, which optionally is the CD28 transmembrane domain, wherein the modification is or includes a substitution of the hydrophobic amino acid with another different hydrophobic amino acid residue; or the one or more modifications is not or does not include a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, which optionally is the CD28 transmembrane domain, other than a substitution with another hydrophobic amino acid residue.

[0019] In some embodiments, the one or more modifications include a modification at an amino acid residue corresponding to an amino acid residue between residues 28 and 42 with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the amino acid

modification is an insertion and the variant chimeric receptor includes an insertion between amino acid residues adjacent to the junction between the domains, which optionally corresponds to amino acid residues 27 and 28 with reference to numbering set forth SEQ ID NO:5. In some embodiments, the variant chimeric receptor includes insertion of 1, 2, 3, 4 or 5 amino acid residues. In some embodiments, the insertion is of any amino acid residue, which, optionally, is asparagine (N).

[0020] In some embodiments, the one or more modifications include an amino acid replacement(s) and the amino acid replacement(s) are at one or more residues selected from amino acid residue 28, 31 or 34 with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the amino acid replacement(s) is to any other amino acid residue, which, optionally, is selected from among leucine (L), asparagine (N), glutamine (Q), alanine (A), serine (S) or histidine (H). In some embodiments, the amino acid replacement(s) is selected from among K28A, K28H, K28L, K28Q, K28S, R31A, R31H, R31L, R31N, R31S, L34A and L34S, with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the amino acid replacement does not include the single amino acid replacement corresponding to L34A or L34S, with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the amino acid replacements are selected from among K28Q/R31A, K28Q/R31N, K28Q/R31S, K28Q/L34A, K28Q/L34S, R31N/L34A, R31N/L34S, K28Q/R31N/L34A, K28Q/R31N/L34S.

[0021] In some embodiments, the variant chimeric receptor includes a modified junction region including less than 100% sequence identity to SEQ ID NO:137 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% to SEQ ID NO:137 and includes the modification(s), such as any described herein; and/or the variant chimeric receptor includes a sequence including less than 100% sequence identity to SEQ ID NO:5 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% to SEQ ID NO:5 and includes the modification(s), such as any described herein.

[0022] In some embodiments, the variant chimeric receptor includes a modified junction region selected from among: i) the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184; ii) a functional variant thereof including a sequence of amino acids that exhibits at least 95%, 96%, 97%, 98% or 99% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184 and that include the modification(s); or iii) a functional portion of i) or ii) and includes the modification(s).

[0023] In some embodiments, the variant chimeric receptor includes i) the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183; ii) a functional variant thereof including a sequence of amino acids that exhibits at least 95%, 96%, 97%, 98%, or 99% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183 and that include the modification(s); ii) or is a functional portion of i) or ii) and includes the modification(s).

[0024] In some embodiments, the modified junction region includes no more than 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications compared to the junction region of the reference chimeric receptor.

[0025] In some embodiments, the first domain or second domain is a transmembrane domain and the corresponding domain in the variant chimeric receptor includes a substantially hydrophobic hydropathy profile and/or has a positive grand average of hydropathy (GRAVY) value. In some embodiments, the GRAVY value is greater than 0, 0.25, 0.5, 0.75, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 or greater.

[0026] In some embodiments, the first domain or second domain includes an intracellular signaling domain and the corresponding domain in the variant chimeric receptor is capable of inducing the activation or cellular localization of a TRAF and/or is capable of inducing TRAF-mediated signaling. In some embodiments, the intracellular signaling domain is a 4-1BB co-stimulatory signaling domain and/or the TRAF is selected from among TRAF1, TRAF2 or TRAF3. In some embodiments, the variant chimeric receptor includes amino acids TTQE at positions corresponding to 49-52 and/or amino acids PEEE at positions corresponding to residues 60-63, each with reference to numbering set forth in SEQ ID NO:5.

[0027] In some embodiments, the HLA is an HLA class I and/or an HLA class II. In some embodiments, the HLA class I is selected from an HLA allele set forth in Table 1A and/or the HLA class II allele is selected from an HLA allele set forth in Table 1B. In some embodiments, the HLA class I allele is selected from among HLA-A*02:01, HLA-A*03:01, HLA-A*11:01 and HLA-B*08:01.

[0028] In some embodiments, the HLA includes a plurality of HLA molecules and average binding affinities to one or more of the plurality of HLA molecules is lower and/or the number of peptide fragments that individually bind to one or more of the plurality of HLA molecules is reduced. In some embodiments, the plurality of HLA molecules is selected from a plurality of HLA class I molecules that represent greater than 50%, greater than 60%, greater than 70%,

greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class I molecules in the worldwide population or in the Caucasian population; a plurality of HLA class II molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class II molecules in the worldwide population or in the Caucasian population; or a plurality of HLA class I molecules and HLA class II molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class I molecules and HLA class II molecules in the worldwide population or in the Caucasian population.

[0029] In some embodiments, the binding affinity is as determined *in vitro*.

[0030] In some embodiments, the variant chimeric receptor exhibits reduced immunogenicity compared to the reference chimeric receptor upon administration to a human subject, optionally wherein the subject has received administration of the reference chimeric receptor. In some embodiments, the reduced immunogenicity includes a reduced CD4+ T cell immune response and/or a reduced CD8+ T cell immune response.

[0031] In some embodiments, the reference chimeric receptor further includes an extracellular ligand-binding domain; and/or the variant chimeric receptor further includes an extracellular ligand-binding domain. In some embodiments, the variant chimeric receptor is a chimeric antigen receptor (CAR), wherein the ligand-binding domain is an antigen-binding domain. In some embodiments, the antigen-binding domain is an antibody or an antibody fragment. In some embodiments, the antigen-binding domain is an antibody fragment that is a single chain fragment. In some embodiments, the fragment includes antibody variable regions joined by a flexible immunoglobulin linker. In some embodiments, the antibody fragment includes an scFv.

[0032] In some embodiments, the ligand-binding domain specifically binds an antigen that is associated with a disease or disorder. In some embodiments, the disease or disorder is an infectious disease or condition, an autoimmune disease, an inflammatory disease or a tumor or a cancer; the ligand-binding domain specifically binds to a tumor antigen; and/or the ligand-binding domain specifically binds to an antigen selected from the group including ROR1, Her2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, EPHA2, ErbB2, ErbB3, ErbB4, FBP, fetal acetylcholine e receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-

13R-alpha2, kdr, kappa light chain, Lewis Y, L1-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, MAGE A3, CE7, Wilms Tumor 1 (WT-1) and cyclin A1 (CCNA1).

[0033] In some embodiments, the reference chimeric receptor further includes an activating cytoplasmic signaling domain; and/or the variant chimeric receptor further includes an activating cytoplasmic domain. In some embodiments, the activating cytoplasmic domain includes a T cell receptor (TCR) component and/or includes an immunoreceptor tyrosine-based activation motif (ITAM). In some embodiments, the activating cytoplasmic signaling domain is or includes a cytoplasmic signaling domain of a zeta chain of a CD3-zeta (CD3 ζ) chain or a functional variant or signaling portion thereof.

[0034] In some embodiments, the reference chimeric receptor includes from its N to C terminus in order: an extracellular ligand-binding domain, the first domain that is a transmembrane domain, the second domain that is an intracellular costimulatory domain and an activating cytoplasmic signaling domain; and/or the variant chimeric receptor includes from its N to C terminus in order: an extracellular ligand-binding domain, a transmembrane domain, a intracellular costimulatory domain and an activating cytoplasmic signaling domain, wherein the transmembrane domain and intracellular costimulatory domain are joined in contiguous sequence at a junction to form the modified junction region.

[0035] In some embodiments, provided herein is a nucleic acid molecule encoding any of the variant chimeric receptor described herein. In some embodiments, provided herein is a vector containing the nucleic acid molecule. In some embodiments, the vector is a viral vector. In some embodiments, the vector is a retroviral vector, which optionally is a lentiviral vector or a gammaretroviral vector.

[0036] In some embodiments, provided herein is an engineered cell including the nucleic acid or the vector of any of the embodiments described herein or expressing the chimeric receptor of any of chimeric receptors described herein. In some embodiments, the engineered cell is a T cell. In some embodiments, the engineered cell is a CD4⁺ T cell and/or a CD8⁺ T cell.

[0037] In some embodiments, provided herein is a composition including the engineered cells of any of the embodiments described herein, and optionally a pharmaceutically acceptable buffer.

[0038] In some embodiments, provided herein is a method of treatment including administering the cell or the composition of any of the embodiments described herein to a subject having a disease or condition. In some embodiments, the chimeric receptor specifically binds to a ligand or antigen associated with the disease or condition. In some embodiments, the disease or condition is a cancer, a tumor, an autoimmune disease or disorder, or an infectious disease.

[0039] In some embodiments, the genetically engineered T cells in the composition exhibit increased or longer expansion and/or persistence in the subject than in a subject administered the same or about the same dosage amount of a reference cell composition expressing the reference chimeric receptor. In some embodiments, the increase is at least 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, or 5-fold. In some embodiments, the increase is observed or is present within a month, within two months, within six months or within one year of administering the cells.

Brief Description of the Drawings

[0040] **FIG. 1** shows results from an exemplary chromium release assay detecting the presence of a cytolytic immune response specific for CAR-expressing cells following administration of anti-CD19 CAR-expressing cells in a human subject. Results are shown for mixed-lymphocyte cultures containing peripheral blood mononuclear cells (PBMCs) derived from the subject pre-infusion (left panel) and post-infusion (right panel) with the CAR-expressing cells, in the presence of either CAR-expressing (“CD19-CAR”) and non-CAR-expressing (“Mock”). “E/T”=effector to target cell ratio.

[0041] **FIG. 2** shows results from an exemplary ELISpot analysis confirming immune responses to certain overlapping peptides representing particular regions of a CAR in an exemplary human subject. Numbers labeled with “pep” represent various overlapping peptides along the length of the CAR sequence, with corresponding regions indicated above the chart.

[0042] **FIG. 3** shows an epitope affinity map for predicted binding affinities of peptides of an exemplary region of a chimeric receptor for binding to HLA-A2:01, including a series of overlapping 8mer to 14mer peptides of an exemplary junction region having an amino acid sequence CYSLLVTVAFIIFWVKRGRKKLLYIFKQPF (SEQ ID NO: 6) where residues 1-15 correspond to an exemplary CD28 transmembrane domain and residues 16-30 correspond to an

exemplary 4-1BB costimulatory domain. The figure also depicts predicted binding affinities of a series of overlapping 8mer to 14mer peptides of a variant junction region having an amino acid sequence CYSLLVTVAFIIFWVNNKRGRKKLLYIFKQPF (SEQ ID NO: 13), containing inserted asparagine residues between the CD28 transmembrane domain and 4-1BB costimulatory domain.

[0043] FIG. 4A and FIG. 4B depict algorithm-based T cell epitope predictions for HLA class I and HLA class II alleles, respectively, showing the total number of sequences in the dataset including each position along the length of the sequence with a predicted IC₅₀ of less than 50nm weighted according to the frequency of the individual HLA alleles in the population.

[0044] FIG. 5 depicts algorithm-based T cell epitope predictions for HLA class I and HLA class II alleles of a series of variant peptides. Scores were determined and weighted as described in Example 2. Triangles and a dotted line indicated class I weighted scores. Circles and a solid line indicate class II weighted scores.

[0045] FIG. 6 depicts the amino acid sequence of an exemplary CD28-4-1BB sequence of SEQ ID NO: 5. Amino acids corresponding to the CD28 transmembrane domain are indicated by a solid line with arrows indicating the beginning and end positions; amino acids of the exemplary 4-1BB costimulatory domain are indicated by a dashed line with arrows indicating the beginning and end positions; amino acids of the exemplary junction region are indicated by a dashed and dotted line with arrows indicating the beginning and end positions and by italics. The two amino acids immediately flanking the junction site are indicated by a box. Exemplary amino acids that in some embodiments are targeted for modification, including K28, R31, and L34, are bolded and underlined. Regions of acidic residues that may be involved in 4-1BB-mediated TRAF-binding and signaling are indicated by a double underline.

[0046] FIG. 7 shows the relative binding to various MHC Class II molecules of overlapping 15-mer peptides within a portion of the region spanning the junction between the CD28 and 4-1BB-derived sequence (set forth in SEQ ID NO: 160; the canonical or “native” sequence) or within a corresponding portion containing either an R31H, R31N, or L34A substitution (with reference to numbering set forth in SEQ ID NO: 5). The REVEAL® score for binding is shown. The score of individual HLA class II alleles are individually labeled with a corresponding letter as follows: A: DPA1*01:03; DPB1*04:01; B: DRA*01:01; DRB1*03:01; C: DRA*01:01; DRB1*15:01; D: DRA*01:01; DRB1*11:01; E: DPA1*01:03; DPB1*04:02; F: DPA1*01:03; DPB1*03:01; G: DPA1*01:03; DPB1*01:01; H: DPA1*02:01; DPB1*01:01; I: DPA1*02:01;

DPB1*04:02; J: DRA*01:01; DRB1*11:04; K: DRA*01:01; DRB1*01:02; and L: DPA1*02:01; DPB1*15:01.

[0047] **FIG. 8** shows a comparison of the *in silico*-predicted versus *in vitro*-determined binding of a 15-mer peptide from the CD28-4-1BB junction to an MHC Class II molecule.

[0048] **FIG. 9A** and **FIG. 9B** show expression of the variant CARs containing a modified junction region on the surface and intracellularly, respectively, in engineered CD4+ T cells. The Y-axis depicts surface expression of the CAR by detection with an anti-idiotypic antibody (Surface ID). The X-axis depicts surface expression of the surrogate EGFRt marker using an anti-EGFR antibody (Erb).

[0049] **FIG. 10** shows mean fluorescence intensity (MFI) as determined by flow cytometry for surface expression of the CARs on both CD4+ and CD8+ T cells.

[0050] **FIG. 11** shows flow cytometry plots depicting intracellular levels of IL-2 and IFN- γ in CD4+/CAR+ cells and CD8+/CAR+ cells.

[0051] **FIG. 12** shows expansion in a serial re-stimulation assay of the anti-CD19 CAR-engineered cells expressing variant CARs containing a modified junction region as compared to cells expressing the CAR with the native junction region.

Detailed Description

[0052] Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as is commonly understood by one of ordinary skill in the art to which the claimed subject matter pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

[0053] All publications, including patent documents, scientific articles and databases, referred to in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication were individually incorporated by reference. If a definition set forth herein is contrary to or otherwise inconsistent with a definition set forth in the patents, applications, published applications and other publications that are herein incorporated by reference, the definition set forth herein prevails over the definition that is incorporated herein by reference.

[0054] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

I. OVERVIEW

[0055] Provided are recombinant receptors, including chimeric receptors, e.g. chimeric antigen receptors, that exhibit one or more amino acid sequence differences compared to a reference chimeric receptor. In some embodiments, the provided variant chimeric receptors contain a modified junction region containing one or more modifications (e.g. amino acid insertions, deletions or amino acid replacements) compared to a junction region of a reference chimeric receptor such that peptide fragments (e.g. of 8 to 24 amino acids in length, e.g. 8 to 15 or 8 to 13 amino acids in length, such as about or 8, 9, 10, 11, 12, 13, 14, or 15 amino acids in length) derived from the modified junction region exhibit lower binding affinity for a human leukocyte antigen (HLA) compared to binding of corresponding peptide fragments derived from the reference junction region for the HLA. In some embodiments, a host immune response, e.g. humoral or cell-mediated, to the variant chimeric receptor following administration to the subject is reduced compared to the reference chimeric receptor. In some embodiments, the reduced immune response is a reduced detectable immune response against a region of the variant chimeric receptor containing the modified junction region.

[0056] In some embodiments, chimeric receptors, such as chimeric antigen receptor, contain one or more domains that combine a ligand-binding domain (e.g. antibody or antibody fragment) that provides specificity for a desired antigen (e.g., tumor antigen) with intracellular signaling domains. In some embodiments, the intracellular signaling domain is an activating intracellular domain portion, such as a T cell activating domain, providing a primary activation signal. In some embodiments, the intracellular signaling domain contains or additionally contains a costimulatory signaling domain to facilitate effector functions. In some embodiments, chimeric receptors when genetically engineered into immune cells can modulate T cell activity, and, in some cases, can modulate T cell differentiation or homeostasis, thereby resulting in genetically engineered cells with improved longevity, survival and/or persistence *in vivo*, such as for use in adoptive cell therapy methods.

[0057] Adoptive cell therapies (including those involving the administration of cells expressing chimeric receptors specific for a disease or disorder of interest, such as chimeric antigen receptors (CARs) and/or other recombinant antigen receptors, as well as other adoptive immune cell and adoptive T cell therapies) can be effective in the treatment of cancer and other

diseases and disorders. In certain contexts, available approaches to adoptive cell therapy may not always be entirely satisfactory. In some contexts, optimal efficacy can depend on the ability of the administered cells to recognize and bind to a target, e.g., target antigen, to traffic, localize to and successfully enter appropriate sites within the subject, tumors, and environments thereof, to become activated, expand, to exert various effector functions, including cytotoxic killing and secretion of various factors such as cytokines, to persist, including long-term, to differentiate, transition or engage in reprogramming into certain phenotypic states (such as effector, long-lived memory, less-differentiated, and effector states), to provide effective and robust recall responses following clearance and re-exposure to target ligand or antigen, and avoid or reduce exhaustion, anergy, terminal differentiation, and/or differentiation into a suppressive state.

[0058] In some cases, adoptive therapy methods are not completely satisfactory in all of these respects. In some aspects, the provided embodiments are based on observations that the efficacy of adoptive cell therapy may be limited by the development of an immune response in the subject to the cells and/or construct administered. For example, in some cases, exposure to a chimeric receptor may be limited by host immune responses against the recombinant receptors expressed by the administered cells, which may prematurely eliminate the cells. It is observed that even in certain subjects having B cell malignancies, who often are immunocompromised, immune responses can be detected that are specific for regions of receptors expressed by cells administered in adoptive cell therapy. For example, as shown herein, subjects administered cells genetically engineered with a CAR developed a specific immune response to an immunogenic region of the chimeric region containing the junction between the transmembrane and costimulatory domain of the CAR.

[0059] In some aspects, the development of an immune response can reduce the exposure or persistence of T cells expressing the chimeric receptor in the course of adoptive therapy methods. Yet, observations indicate that, in some cases, increased exposure of the subject to administered cells expressing the recombinant receptors (e.g., increased number of cells or duration over time) may improve efficacy and therapeutic outcomes in adoptive cell therapy. Preliminary analysis conducted following the administration of different CD19-targeting CAR-expressing T cells to subjects with various CD19-expressing cancers in multiple clinical trials revealed a correlation between greater and/or longer degree of exposure to the CAR-expressing cells and treatment outcomes. Such outcomes included patient survival and remission, even in individuals with severe or significant tumor burden.

[0060] Further, in some cases, once such a host immune response develops, either acquired or innate, it may not be feasible or effective to attempt to increase exposure or provide retreatment of subjects by administering a subsequent dose of cells expressing the same recombinant receptor. Once such an immune response has developed against the receptor, administration of such a second or subsequent dose of cells expressing the same receptor or one with similar immunogenic epitopes may result in rapid elimination of the cells before they have had a chance to expand and/or persist to an effective or substantial degree. Thus, it would be advantageous to minimize unwanted immune responses that may destroy or interfere with the activity of engineered cells used in adoptive cell therapy methods, thereby diminishing the effectiveness of the treatment.

[0061] Generally, chimeric receptors include a plurality of different domains, such as a plurality of domains present in molecules endogenous to a subject. For example, CARs are molecules that include an extracellular antigen-recognition domain that specifically binds to a target antigen and an intracellular signaling domain comprising an ITAM (e.g. CD3-zeta intracellular signaling domain). In some embodiments, the extracellular antigen-recognition domain comprises an antibody or antigen-binding fragment (e.g. scFv). A CAR also can contain a transmembrane domain and/or endodomain between the extracellular recognition domain and signaling domain, which can include transmembrane and/or intracellular signaling domains of a co-stimulatory molecule, such as a CD28 or 4-1BB co-stimulatory molecule. In some cases, such domains can be connected by linkers.

[0062] In some cases, the plurality of domains are not normally present adjacent to each other in sequence in an endogenous molecule in a subject. For example, in some embodiments, a first domain and a second domain of a chimeric receptor that are joined at a junction can create a contiguous sequence of amino acids that may not be present in a subject, particularly where such two domains do not naturally occur adjacent together in the same protein and/or are separated by a synthetic linker. In some cases, this can result in the presence of a non-native sequence that is not identical to a sequence present in an endogenous molecule of the host in the portion of the sequence of a chimeric receptor, such as a CAR, containing the junction between two domains.

[0063] In some cases, a junction region that contains potential peptide epitopes spanning the junction of the two domains can be immunogenic and result in the generation of an immune response upon administration to a subject of a chimeric receptor containing the junction region.

In some embodiments, the junction region can include, e.g. once processed in connection with antigen presentation for display on an HLA molecule, a plurality of individual overlapping peptide fragments of contiguous sequence of about 8 to 24 amino (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) directly C-terminal of the junction that joins a first domain and a second domain of the chimeric receptor and/or of about 8 to 24 amino acids (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) directly N-terminal of the junction, which peptide fragments each can include or span the junction of the two domains. Thus, in some cases, the junction region can contain a plurality of potential peptide epitopes that may exhibit a binding affinity for an HLA molecule and/or be capable of inducing an immune response.

[0064] In some embodiments, the peptide fragment is recognized by the immune response to induce or initiate a detectable immune response, e.g. humoral or cell-mediated, against such peptide sequence. In some embodiments, the peptide fragment can associate or bind with an MHC molecule expressed on a cell for recognition by a T cell receptor (TCR). In some embodiments, the peptide epitope, under certain conditions, can elicit an immune response in an animal, such as a T cell response. For example, in some cases, T cells expressing a TCR that recognizes a T cell epitope in the context of an MHC molecule can become stimulated, thereby leading to T cell responses, such as T cell proliferation, lymphokine secretion, cytotoxic responses, local inflammatory reactions, recruitment of additional immune cells and /or activation of B cells leading to production of antibodies against the protein containing the peptide epitope, such as a chimeric receptor. It is within the level of a skilled artisan to identify a peptide epitope of any chimeric receptor.

[0065] In some embodiments, the provided methods reduce or lessen an immune response in a subject administered with cells expressing the variant chimeric receptor, such as CAR, compared to the immune response generated in the subject administered with cells expressing the reference (unmodified) chimeric receptor, such as reference or unmodified CAR. In some embodiments, the subject does not exhibit or exhibits a reduced immune response or a particular type or degree of immune response, against the variant chimeric receptor or a modified junction region of the chimeric receptor, such as following the administration of the cells expressing the receptor. The type of immune response may be a detectable immune response, a humoral immune response, and/or a cell-mediated immune response.

[0066] In some embodiments, the provided methods achieve increased persistence of genetically engineered T cells expressing the variant chimeric receptor, such as a variant CAR, when administered to a subject. In some embodiments, a genetically engineered cell with increased persistence exhibits better potency in a subject to which it is administered. In some embodiments, the persistence of genetically engineered cells expressing the variant chimeric receptor, such as variant CAR-expressing T cells, in the subject upon administration is greater as compared to that which would be achieved by alternative methods, such as those involving administration of genetically engineered cells expressing the reference or unmodified chimeric receptor. In some aspects, the persistence of administered cells is increased at least or about at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold or more.

[0067] In some embodiments, the degree or extent of persistence of administered cells can be detected or quantified after administration to a subject. For example, in some aspects, quantitative PCR (qPCR) is used to assess the quantity of cells expressing the chimeric receptor (e.g., CAR-expressing cells) in the blood or serum or organ or tissue (e.g., disease site) of the subject. In some aspects, persistence is quantified as copies of DNA or plasmid encoding the receptor, e.g., CAR, per microgram of DNA, or as the number of receptor-expressing, e.g., CAR-expressing, cells per microliter of the sample, e.g., of blood or serum, or per total number of peripheral blood mononuclear cells (PBMCs) or white blood cells or T cells per microliter of the sample. In some embodiments, flow cytometric assays detecting cells expressing the receptor generally using antibodies specific for the receptors also can be performed. Cell-based assays may also be used to detect the number or percentage of functional cells, such as cells capable of binding to and/or neutralizing and/or inducing responses, e.g., cytotoxic responses, against cells of the disease or condition or expressing the antigen recognized by the receptor. In any of such embodiments, the extent or level of expression of another marker associated with the recombinant receptor (e.g. CAR-expressing cells) can be used to distinguish the administered cells from endogenous cells in a subject.

[0068] Also provided are compositions containing the variant chimeric receptor or engineered cells expressing such variant chimeric receptor. Also provided are methods for administering such variant chimeric receptors and compositions to subjects, including cells expressing the variant chimeric receptors and compositions of such cells to subjects, such as for adoptive cell therapy.

II. VARIANT CHIMERIC RECEPTORS

[0069] Provided herein are variant chimeric receptors that contain a modified junction region compared to a junction region of a reference chimeric receptor. In some embodiments, the junction region is a region containing a contiguous sequence of amino acids on each side of a junction between a first and second domain. In some embodiments, by virtue of one or more modifications present in the modified junction region, the variant chimeric receptor contains a domain corresponding to the first domain of the reference chimeric receptor and/or a domain corresponding to the second domain of the reference chimeric receptor that is modified by one or more amino acid differences (e.g. mutations).

[0070] In some embodiments, the variant chimeric receptor contains a modified junction region compared to a junction region of a reference chimeric receptor in which one or more amino acid residues at a position 8 to 24 amino acids (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) directly C-terminal of the junction that joins a first domain and a second domain of the reference chimeric receptor and/or at a position 8 to 24 amino acids (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) directly N-terminal of the junction are modified, such as by insertion, deletion or amino acid replacement. In some embodiments, the variant chimeric receptor contains up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid differences or modifications in the modified junction region compared to the junction region in the reference chimeric receptor.

[0071] In some embodiments, the variant chimeric receptor contains a domain of at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the first domain of the reference chimeric receptor and/or contains a domain of at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the second domain of the reference chimeric receptor. In some embodiments, the variant chimeric receptor contains a domain that is identical in sequence to the first domain of the reference chimeric receptor and contains a domain of at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the second domain of the reference chimeric receptor. In some embodiments, the variant chimeric receptor contains a domain of at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the first domain of the reference chimeric receptor and contains a domain that is identical in

sequence to the second domain of the reference chimeric receptor. In some embodiments, at least one or both of the domains present in the variant chimeric receptor is modified compared to the first domain and/or the second domain of the reference chimeric receptor in the portion containing the modified junction region.

[0072] In some embodiments, the variant chimeric receptor has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the reference chimeric receptor. In some embodiments, the variant chimeric receptor contains up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid differences or modifications (e.g. amino acid insertions, deletions or replacements) compared to the reference chimeric receptor.

[0073] In some embodiments, the first and/or second domain of the reference chimeric receptor (e.g. reference CAR) is a domain of a natural endogenous human protein or a domain having 100% identity with a domain or function portion thereof of a natural or endogenous protein. In some embodiments, the first domain and second domain are not present in the same molecule *in vivo* in a human subject. In some embodiments, the first domain and second domain are not present in a single natural or endogenous human protein or polypeptide.

[0074] In some embodiments, the first and/or second domain is or comprises an extracellular binding domain, a hinge domain, a transmembrane domain, or an intracellular signaling domain or functional portions thereof. In some embodiments, the intracellular signaling domain is or comprises a costimulatory signaling domain, such as a CD28, 4-1BB, or ICOS co-stimulatory signaling domain. In some embodiments, the intracellular signaling domain is or comprises an activating cytoplasmic signaling domain, such as a domain that is or includes a T cell receptor (TCR) component and/or that contains an immunoreceptor tyrosine-based activation motif (ITAM). In some cases, the activating cytoplasmic domain is or comprises a cytoplasmic signaling domain of a zeta chain of a CD3-zeta (CD3 ζ) chain or a functional variant or signaling portion thereof.

[0075] In some embodiments, the reference chimeric receptor is a CAR. In some embodiments, the chimeric receptors, such as a CAR, contains from its N-terminus to C-terminus in order: an extracellular ligand-binding domain, a transmembrane domain and an intracellular signaling domain. In some embodiments, the intracellular signaling domain is or includes an activating signaling domain (e.g. a components of TCR and/or containing an ITAM, for example a CD3-zeta signaling domain). In some embodiments, the intracellular signaling

domain is or includes a costimulatory signaling domain (e.g. a CD28, 4-1BB or ICOS signaling domain). In some embodiments, the intracellular signaling domain contains only one of the costimulatory signaling domain or activating signaling domain or contains both domains in either order. In some embodiments, the intracellular signaling domain contains both the costimulatory signaling domain and activating signaling domain.

[0076] In some embodiments, the reference chimeric receptor contains a junction region of a first and second domain that is immunogenic. In some embodiments, the immunogenic region includes one or more peptide epitopes (also called a T cell epitope). In some cases, a junction region that contains potential peptide epitopes spanning the junction of the two domains can be immunogenic and result in the generation of an immune response upon administration to a subject of a chimeric receptor containing the junction region. In some embodiments, the junction region can include, e.g. once processed in connection with antigen presentation for display on an HLA molecule, a plurality of individual overlapping peptide fragments of contiguous sequence of about 8 to 24 amino (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) directly C-terminal of the junction that joins a first domain and a second domain of the chimeric receptor and/or of about 8 to 24 amino acids (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) directly N-terminal of the junction, which peptide fragments each can include or span the junction of the two domains. Thus, in some cases, the junction region can contain a plurality of potential peptide epitopes that may exhibit a binding affinity for an HLA molecule and/or be capable of inducing an immune response.

[0077] In some embodiments, an immunogenic region, such as a junction region, of a chimeric receptor can be identified. In some embodiments, the immunogenic region can be identified by its ability to bind to an MHC molecule or by its ability to elicit an immune response under certain conditions. In some embodiments, overlapping peptides of a chimeric receptor, such as overlapping 8mer to 20 mer peptides, such as 9mers, 10mers, 11mers, 12mers, 13mers, 14mers or 15mers can be assessed for MHC binding using algorithmic or other computational methods, such as described below. In some embodiments, a chimeric receptor can be assessed to determine if it is immunogenic by assessing an immune response in a subject to which it has been administered, such as a subject administered cells genetically engineered with the chimeric receptor (e.g. CAR). Exemplary methods of assessing immune responses are described below.

[0078] In some embodiments, the at least one peptide epitope is capable of binding to a major histocompatibility complex (MHC) molecule, such as a class I or class II protein, which are molecules that contain a polymorphic peptide binding site or binding groove that can, in some cases, complex with peptide fragments of polypeptides, including peptides processed by the cell machinery. In some embodiments, the peptide epitope is capable of binding to an MHC molecule that is a human MHC molecule. In some embodiments, the MHC molecule is a human leukocyte antigen (HLA) molecule. In some embodiments, the at least one peptide epitope exhibits a binding affinity (e.g. IC50) for an HLA molecule, such as an HLA class I molecule or an HLA class II molecule. In some embodiments, the junction region of the reference chimeric receptor contains a peptide epitope that exhibits a binding affinity of less than 1000 nM, less than 500 nM or less than 50 nM.

[0079] In some embodiments, at least one or more peptide epitopes of a junction region of a reference chimeric receptor is an MHC class II epitope. In some embodiments, peptides that bind to MHC class II molecules can be between 8 and 20 amino acids in length, including between 10 and 17 amino acids in length, such as or about at least or at least or about 10, 11, 12, 13, 14, 15, 16 or 17 amino acids in length, for example about 15 amino acids in length.. In some embodiments, the peptides that bind to MHC class II molecules can be longer than 20 amino acids. In some embodiments, the peptide lies in an extended conformation along the MHC II peptide-binding groove. In some embodiments, the MHC II peptide-binding groove is open at both ends. In some embodiments, the peptide is held in place at least in part by main-chain atom contacts with conserved residues that line the peptide-binding groove. In some embodiments, the MHC class II is an $\alpha\beta$ dimer that can be or include any MHC class II allelic protein known to be present in a subject, such as a human subject. In some embodiments, the MHC allele can be, but is not limited to, DR1 , DR3, DR4, DR7, DR52, DQ1 , DQ2, DQ4, DQ8 and DP1. In some embodiments, the MHC class II allele can be any set forth in Tables 1B, including isotype matched alpha and beta associations or mixed-isotype heterodimers combining alpha and beta chains of different alleles. In some embodiments, the MHC class II can be a dimer that is or includes an alpha and/or beta allele from among HLA-DPA1*0103, HLA-DPA1*0201, HLA-DPB1*0101, HLA-DPB1*0301, HLA-DPB1*0401, HLA-DPB*0402, HLA-DPB1*1501, HLA-DRA*0101, HLA-DRB1*0101, HLA-DRB1*0102, HLA-DRB*0301, HLA-DRB*0701, HLA-DRB*0401, HLA-DRB1*1101, HLA-DRB1*1104, HLA-DRB*1501, and/or HLA-DQB1*0201.

[0080] In some embodiments, the at least one peptide epitope of a junction region of a reference chimeric receptor is an MHC class I epitope. In some embodiments, peptides that bind to MHC class I molecules can be between 7 to 15 amino acids in length. In some embodiments, peptides that bind to MHC class I molecule can be between 8 to 13 amino acids in length. In some embodiments, the binding of the peptide is stabilized at its two ends by contacts between atoms in the main chain of the peptide and invariant sites in the peptide-binding groove of all MHC class I molecules. In some embodiments, there are invariant sites at both ends of the groove which bind the amino and carboxy termini of the peptide. In some embodiments, variations in peptide length can be accommodated by a kink in the peptide backbone. In some embodiments, the kink includes proline or glycine residues, which may allow flexibility. In some embodiments, the MHC class I allele can be any known to be present in a subject, such as a human subject. In some embodiments, the MHC class I allele is an HLA-A2, HLA-A1, HLA-A3, HLA-A24, HLA-A28, HLA-A31, HLA-A33, HLA-A34, HLA-B7, HLA-B45 or HLA-Cw8 allele. In some embodiments, the MHC class I allele can be any set forth in Tables 1A, which are among the most frequent MHC class I alleles (Solberg et al., (2008) *Hum Immunol.* 2008 Jul; 69(7):443-6). In some embodiments, the HLA class I allele is HLA-A*02:01, HLA-A*03:01, HLA-A*11:01 or HLA-B*08:01.

[0081] In some embodiments, the MHC class I allele is an HLA-A2 allele, which in some populations is expressed by approximately 50% of the population. In some embodiments, the HLA-A2 allele can be an HLA-A*0201, *0202, *0203, *0206, or *0207 gene product. In some cases, there can be differences in the frequency of subtypes between different populations. For example, in some embodiments, more than 95% of the HLA-A2 positive Caucasian population is HLA-A*0201, whereas in the Chinese population the frequency has been reported to be approximately 23% HLA-A*0201, 45% HLA-A*0207, 8% HLA-A*0206 and 23% HLA-A*0203. In some embodiments, the MHC molecule is HLA-A*0201.

[0082] In some embodiments, the variant chimeric receptor can contain from its N-terminus to C-terminus in order: an extracellular ligand-binding domain, a transmembrane domain and an intracellular signaling domain, which optionally can include a costimulatory signaling domain (e.g. CD28, 4-1BB or ICOS) and/or an activating signaling domain (e.g. a components of TCR and/or containing an ITAM, for example a CD3-zeta signaling domain) each alone as part of the intracellular signaling domain or in either order, in which the variant chimeric receptor contains a modification at one or more amino acid residues within a contiguous portion of 8 to 24 amino

acids (e.g. 8 to 15 amino acids or 8 to 13 amino acids, such as about or 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids) on either side (N-terminal and/or C-terminal) of the junction.

[0083] In some embodiments, the features of a reference chimeric receptor can be any described in subsection 1 below. In some embodiments, the features of a variant chimeric receptor also can be any as described in subsection 1 below, except that the variant chimeric receptor contains one or more modifications (e.g. insertions, deletions or replacements) in a modified junction region as described.

1. *Chimeric Receptors*

[0084] Exemplary recombinant receptors, including CARs and recombinant TCRs, as well as methods for engineering and introducing the receptors into cells, include those described, for example, in international patent application publication numbers WO200014257, WO2013126726, WO2012/129514, WO2014031687, WO2013/166321, WO2013/071154, WO2013/123061 U.S. patent application publication numbers US2002131960, US2013287748, US20130149337, U.S. Patent Nos.: 6,451,995, 7,446,190, 8,252,592, , 8,339,645, 8,398,282, 7,446,179, 6,410,319, 7,070,995, 7,265,209, 7,354,762, 7,446,191, 8,324,353, and 8,479,118, and European patent application number EP2537416, and/or those described by Sadelain et al., *Cancer Discov.* 2013 April; 3(4): 388–398; Davila et al. (2013) *PLoS ONE* 8(4): e61338; Turtle et al., *Curr. Opin. Immunol.*, 2012 October; 24(5): 633-39; Wu et al., *Cancer*, 2012 March 18(2): 160-75. In some embodiments, the genetically engineered antigen receptors include a CAR as described in U.S. Patent No.: 7,446,190, and those described in International Patent Application Publication No.: WO/2014055668 A1. In some embodiments, similar methods for the construction and introduction or transfer into immune cells can be employed for the provided chimeric receptors.

[0085] In some embodiments, the chimeric receptor includes a ligand-binding domain that binds, such as specifically binds, to an antigen (or a ligand). Among the antigens targeted by chimeric receptors are those expressed in the context of a disease, condition, or cell type to be targeted via the adoptive cell therapy. Among the diseases and conditions are proliferative, neoplastic, and malignant diseases and disorders, including cancers and tumors, including hematologic cancers, cancers of the immune system, such as lymphomas, leukemias, and/or myelomas, such as B, T, and myeloid leukemias, lymphomas, and multiple myelomas. In some embodiments, the antigen (or a ligand) is a polypeptide. In some embodiments, it is a

carbohydrate or other molecule. In some embodiments, the antigen (or a ligand) is selectively expressed or overexpressed on cells of the disease or condition, *e.g.*, the tumor or pathogenic cells, as compared to normal or non-targeted cells or tissues. In other embodiments, the antigen is expressed on normal cells and/or is expressed on the engineered cells. In some embodiments, the antigen (or a ligand) is a tumor antigen or cancer marker. In some embodiments, the antigen (or a ligand) is or includes orphan tyrosine kinase receptor ROR1, tEGFR, Her2, L1-CAM, CD19, CD20, CD22, BCMA, mesothelin, CEA, and hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, EPHA2, ErbB2, 3, or 4, FBP, fetal acetylcholine receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, Lewis Y, L1-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, ROR1, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, Her2/neu, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, and MAGE A3, CE7, Wilms Tumor 1 (WT-1), a cyclin, such as cyclin A1 (CCNA1), and/or biotinylated molecules, and/or molecules expressed by HIV, HCV, HBV or other pathogens. In some embodiments, the antigen is a pathogen-specific antigen. In some embodiments, the antigen is a viral antigen (such as a viral antigen from HIV, HCV, HBV, etc.), bacterial antigens, and/or parasitic antigens.

[0086] In some embodiments, the chimeric receptor is constructed with a specificity for a particular antigen (or marker or ligand), such as an antigen expressed in a particular cell type to be targeted by adoptive therapy, *e.g.*, a cancer marker, and/or an antigen intended to induce a dampening response, such as an antigen expressed on a normal or non-diseased cell type. Thus, the chimeric receptor typically includes in its extracellular portion one or more ligand binding domains.

[0087] In some embodiments, the chimeric receptor is a CAR. In some embodiments, the CAR typically includes in its extracellular portion one or more antigen binding molecules, such as one or more antigen-binding fragment, domain, or portion, or one or more antibody variable domains, and/or antibody molecules. In some embodiments, the CAR includes an antigen-binding portion or portions of an antibody molecule, such as a single-chain antibody fragment (scFv) derived from the variable heavy (VH) and variable light (VL) chains of a monoclonal antibody (mAb).

[0088] The term “antibody” herein is used in the broadest sense and includes polyclonal and monoclonal antibodies, including intact antibodies and functional (antigen-binding) antibody fragments, including fragment antigen binding (Fab) fragments, $F(ab')_2$ fragments, Fab' fragments, Fv fragments, recombinant IgG (rIgG) fragments, variable heavy chain (V_H) regions capable of specifically binding the antigen, single chain antibody fragments, including single chain variable fragments (scFv), and single domain antibodies (*e.g.*, sdAb, sdFv, nanobody) fragments. The term encompasses genetically engineered and/or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, fully human antibodies, humanized antibodies, and heteroconjugate antibodies, multispecific, *e.g.*, bispecific, antibodies, diabodies, triabodies, and tetrabodies, tandem di-scFv, tandem tri-scFv. Unless otherwise stated, the term “antibody” should be understood to encompass functional antibody fragments thereof. The term also encompasses intact or full-length antibodies, including antibodies of any class or sub-class, including IgG and sub-classes thereof, IgM, IgE, IgA, and IgD.

[0089] In some embodiments, the antigen-binding proteins, antibodies and antigen binding fragments thereof specifically recognize an antigen of a full-length antibody. In some embodiments, the heavy and light chains of an antibody can be full-length or can be an antigen-binding portion (a Fab, $F(ab')_2$, Fv or a single chain Fv fragment (scFv)). In other embodiments, the antibody heavy chain constant region is chosen from, *e.g.*, IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD, and IgE, particularly chosen from, *e.g.*, IgG1, IgG2, IgG3, and IgG4, more particularly, IgG1 (*e.g.*, human IgG1). In another embodiment, the antibody light chain constant region is chosen from, *e.g.*, kappa or lambda, particularly kappa.

[0090] Among the provided antibodies are antibody fragments. An “antibody fragment” refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; linear antibodies; variable heavy chain (V_H) regions, single-chain antibody molecules such as scFvs and single-domain V_H single antibodies; and multispecific antibodies formed from antibody fragments. In particular embodiments, the antibodies are single-chain antibody fragments comprising a variable heavy chain region and/or a variable light chain region, such as scFvs.

[0091] The term “variable region” or “variable domain” refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable domains of the heavy chain and light chain (V_H and V_L , respectively) of a native antibody generally have

similar structures, with each domain comprising four conserved framework regions (FRs) and three CDRs. (See, *e.g.*, Kindt et al. *Kuby Immunology*, 6th ed., W.H. Freeman and Co., page 91 (2007)). A single V_H or V_L domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a V_H or V_L domain from an antibody that binds the antigen to screen a library of complementary V_L or V_H domains, respectively. See, *e.g.*, Portolano et al., *J. Immunol.* 150:880-887 (1993); Clarkson et al., *Nature* 352:624-628 (1991).

[0092] Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody. In some embodiments, the CAR comprises an antibody heavy chain domain that specifically binds the antigen, such as a cancer marker or cell surface antigen of a cell or disease to be targeted, such as a tumor cell or a cancer cell, such as any of the target antigens described herein or known in the art.

[0093] Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells. In some embodiments, the antibodies are recombinantly-produced fragments, such as fragments comprising arrangements that do not occur naturally, such as those with two or more antibody regions or chains joined by synthetic linkers, *e.g.*, peptide linkers, and/or that are may not be produced by enzyme digestion of a naturally-occurring intact antibody. In some embodiments, the antibody fragments are scFvs.

[0094] A “humanized” antibody is an antibody in which all or substantially all CDR amino acid residues are derived from non-human CDRs and all or substantially all FR amino acid residues are derived from human FRs. A humanized antibody optionally may include at least a portion of an antibody constant region derived from a human antibody. A “humanized form” of a non-human antibody, refers to a variant of the non-human antibody that has undergone humanization, typically to reduce immunogenicity to humans, while retaining the specificity and affinity of the parental non-human antibody. In some embodiments, some FR residues in a humanized antibody are substituted with corresponding residues from a non-human antibody (*e.g.*, the antibody from which the CDR residues are derived), *e.g.*, to restore or improve antibody specificity or affinity.

[0095] In some embodiments, the CAR contains an antibody or an antigen-binding fragment (*e.g.* scFv) that specifically recognizes an antigen, such as an intact antigen, expressed on the surface of a cell.

[0096] In some embodiments, the CAR contains a TCR-like antibody, such as an antibody or an antigen-binding fragment (*e.g.* scFv) that specifically recognizes an intracellular antigen, such as a tumor-associated antigen, presented on the cell surface as a MHC-peptide complex. In some embodiments, an antibody or antigen-binding portion thereof that recognizes an MHC-peptide complex can be expressed on cells as part of a recombinant receptor, such as an antigen receptor. Among the antigen receptors are functional non-TCR antigen receptors, such as chimeric antigen receptors (CARs). Generally, a CAR containing an antibody or antigen-binding fragment that exhibits TCR-like specificity directed against peptide-MHC complexes also may be referred to as a TCR-like CAR.

[0097] In some embodiments, an antibody or antigen-binding portion thereof that specifically binds to a MHC-peptide complex, can be produced by immunizing a host with an effective amount of an immunogen containing a specific MHC-peptide complex. In some cases, the peptide of the MHC-peptide complex is an epitope of antigen capable of binding to the MHC, such as a tumor antigen, for example a universal tumor antigen, myeloma antigen or other antigen as described below. In some embodiments, an effective amount of the immunogen is then administered to a host for eliciting an immune response, wherein the immunogen retains a three-dimensional form thereof for a period of time sufficient to elicit an immune response against the three-dimensional presentation of the peptide in the binding groove of the MHC molecule. Serum collected from the host is then assayed to determine if desired antibodies that recognize a three-dimensional presentation of the peptide in the binding groove of the MHC molecule is being produced. In some embodiments, the produced antibodies can be assessed to confirm that the antibody can differentiate the MHC-peptide complex from the MHC molecule alone, the peptide of interest alone, and a complex of MHC and irrelevant peptide. The desired antibodies can then be isolated.

[0098] In some embodiments, an antibody or antigen-binding portion thereof that specifically binds to an MHC-peptide complex can be produced by employing antibody library display methods, such as phage antibody libraries. In some embodiments, phage display libraries of mutant Fab, scFV or other antibody forms can be generated, for example, in which members of the library are mutated at one or more residues of a CDR or CDRs. Exemplary of such

methods are known in the art (see *e.g.* US published application No. US20020150914, US2014/0294841; and Cohen CJ. *et al.* (2003) *J Mol. Recogn.* 16:324-332).

[0099] In some embodiments, the extracellular portion of the CAR, such as an antibody portion thereof, further includes a spacer, such as a spacer region between the antigen-recognition component, *e.g.* scFv, and a transmembrane domain. The spacer may be or include at least a portion of an immunoglobulin constant region or variant or modified version thereof, such as a hinge region, *e.g.*, an IgG4 hinge region, and/or a CH1/CL and/or Fc region. In some embodiments, the constant region or portion is of a human IgG, such as IgG4 or IgG1. The spacer can be of a length that provides for increased responsiveness of the cell following antigen binding, as compared to in the absence of the spacer. In some examples, the spacer is at or about 12 amino acids in length or is no more than 12 amino acids in length. Exemplary spacers include those having at least about 10 to 229 amino acids, about 10 to 200 amino acids, about 10 to 175 amino acids, about 10 to 150 amino acids, about 10 to 125 amino acids, about 10 to 100 amino acids, about 10 to 75 amino acids, about 10 to 50 amino acids, about 10 to 40 amino acids, about 10 to 30 amino acids, about 10 to 20 amino acids, or about 10 to 15 amino acids, and including any integer between the endpoints of any of the listed ranges. In some embodiments, a spacer region has about 12 amino acids or less, about 119 amino acids or less, or about 229 amino acids or less. Exemplary spacers include IgG4 hinge alone, IgG4 hinge linked to CH2 and CH3 domains, or IgG4 hinge linked to the CH3 domain. Exemplary spacers include, but are not limited to, those described in Hudecek *et al.* (2013) *Clin. Cancer Res.*, 19:3153, international patent application publication number WO2014031687, or U.S. Patent No. 8,822,647 or published app. No. US2014/0271635.

[0100] In some embodiments, the constant region or portion is of a human IgG, such as IgG4 or IgG1. In some embodiments, the spacer has the sequence ESKYGPPCPPCP (set forth in SEQ ID NO: 1), and is encoded by the sequence set forth in SEQ ID NO: 158. In some embodiments, the spacer has the sequence set forth in SEQ ID NO: 107. In some embodiments, the spacer has the sequence set forth in SEQ ID NO: 108. In some embodiments, the constant region or portion is of IgD. In some embodiments, the spacer has the sequence set forth in SEQ ID NO: 109. In some embodiments, the spacer has a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS: 1, 107, 108 or 109.

[0101] The extracellular ligand binding, such as antigen recognition domain, generally is linked to one or more intracellular signaling components, such as signaling components that mimic activation through an antigen receptor complex, such as a TCR complex, in the case of a CAR, and/or signal via another cell surface receptor. In some embodiments, a transmembrane domain links the extracellular ligand binding and intracellular signaling domains. In some embodiments, the CAR includes a transmembrane domain fused to the extracellular domain. In one embodiment, a transmembrane domain that naturally is associated with one of the domains in the receptor, e.g., CAR, is used. In some instances, the transmembrane domain is selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins to minimize interactions with other members of the receptor complex.

[0102] The transmembrane domain in some embodiments is derived either from a natural or from a synthetic source. Where the source is natural, the domain in some aspects is derived from any membrane-bound or transmembrane protein. Transmembrane regions include those derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD 154 and/or transmembrane regions containing functional variants thereof such as those retaining a substantial portion of the structural, e.g., transmembrane, properties thereof. In some embodiments, the transmembrane domain is a transmembrane domain derived from CD4, CD28, or CD8, e.g., CD8alpha, or functional variant thereof. Alternatively the transmembrane domain in some embodiments is synthetic. In some aspects, the synthetic transmembrane domain comprises predominantly hydrophobic residues such as leucine and valine. In some aspects, a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. In some embodiments, the linkage is by linkers, spacers, and/or transmembrane domain(s).

[0103] Among the intracellular signaling domains are those that mimic or approximate a signal through a natural antigen receptor, a signal through such a receptor in combination with a costimulatory receptor, and/or a signal through a costimulatory receptor alone. In some embodiments, a short oligo- or polypeptide linker, for example, a linker of between 2 and 10 amino acids in length, such as one containing glycines and serines, e.g., glycine-serine doublet, is present and forms a linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR.

[0104] The receptor, e.g., the CAR, generally includes at least one intracellular signaling component or components. In some embodiments, the receptor includes an intracellular component of a TCR complex, such as a TCR CD3 chain that mediates T-cell activation and cytotoxicity, e.g., CD3 zeta chain. Thus, in some aspects, the antigen-binding portion is linked to one or more cell signaling modules. In some embodiments, cell signaling modules include CD3 transmembrane domain, CD3 intracellular signaling domains, and/or other CD transmembrane domains. In some embodiments, the receptor, e.g., CAR, further includes a portion of one or more additional molecules such as Fc receptor γ , CD8, CD4, CD25, or CD16. For example, in some aspects, the CAR or other chimeric receptor includes a chimeric molecule between CD3-zeta (CD3- ζ) or Fc receptor γ and CD8, CD4, CD25 or CD16.

[0105] In some embodiments, upon ligation of the CAR or other chimeric receptor, the cytoplasmic domain or intracellular signaling domain of the receptor activates at least one of the normal effector functions or responses of the immune cell, e.g., T cell engineered to express the CAR. For example, in some contexts, the CAR induces a function of a T cell such as cytolytic activity or T-helper activity, such as secretion of cytokines or other factors. In some embodiments, a truncated portion of an intracellular signaling domain of an antigen receptor component or costimulatory molecule is used in place of an intact immunostimulatory chain, for example, if it transduces the effector function signal. In some embodiments, the intracellular signaling domain or domains include the cytoplasmic sequences of the T cell receptor (TCR), and in some aspects also those of co-receptors that in the natural context act in concert with such receptors to initiate signal transduction following antigen receptor engagement.

[0106] In the context of a natural TCR, full activation generally requires not only signaling through the TCR, but also a costimulatory signal. Thus, in some embodiments, to promote full activation, a component for generating secondary or co-stimulatory signal is also included in the CAR. In other embodiments, the CAR does not include a component for generating a costimulatory signal. In some aspects, an additional CAR is expressed in the same cell and provides the component for generating the secondary or costimulatory signal.

[0107] T cell activation is in some aspects described as being mediated by two classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR (primary cytoplasmic signaling sequences), and those that act in an antigen-independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic

signaling sequences). In some aspects, the CAR includes one or both of such signaling components.

[0108] In some aspects, the CAR includes a primary cytoplasmic signaling sequence that regulates primary activation of the TCR complex. Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing primary cytoplasmic signaling sequences include those derived from TCR zeta, FcR gamma, CD3 gamma, CD3 delta and CD3 epsilon. In some embodiments, cytoplasmic signaling molecule(s) in the CAR contain(s) a cytoplasmic signaling domain, portion thereof, or sequence derived from CD3 zeta.

[0109] In some embodiments, the CAR includes a signaling domain and/or transmembrane portion of a costimulatory receptor, such as CD28, 4-1BB, OX40, DAP10, and ICOS. In some aspects, the same CAR includes both the activating and costimulatory components.

[0110] In some embodiments, the activating domain is included within one CAR, whereas the costimulatory component is provided by another CAR recognizing another antigen. In some embodiments, the CARs include activating or stimulatory CARs, costimulatory CARs, both expressed on the same cell (see WO2014/055668). In some aspects, the cells include one or more stimulatory or activating CAR and/or a costimulatory CAR. In some embodiments, the cells further include inhibitory CARs (iCARs, see Fedorov et al., *Sci. Transl. Medicine*, 5(215) (December, 2013), such as a CAR recognizing an antigen other than the one associated with and/or specific for the disease or condition whereby an activating signal delivered through the disease-targeting CAR is diminished or inhibited by binding of the inhibitory CAR to its ligand, e.g., to reduce off-target effects.

[0111] In some embodiments, the intracellular signaling component of the recombinant receptor, such as CAR, comprises a CD3 zeta intracellular domain and a costimulatory signaling region. In certain embodiments, the intracellular signaling domain comprises a CD28 transmembrane and signaling domain linked to a CD3 (e.g., CD3-zeta) intracellular domain. In some embodiments, the intracellular signaling domain comprises a chimeric CD28 and CD137 (4-1BB, TNFRSF9) co-stimulatory domains, linked to a CD3 zeta intracellular domain.

[0112] In some embodiments, the CAR encompasses one or more, e.g., two or more, costimulatory domains and an activation domain, e.g., primary activation domain, in the cytoplasmic portion. Exemplary CARs include intracellular components of CD3-zeta, CD28, and 4-1BB.

[0113] In some embodiments, the CAR or other antigen receptor further includes a marker, such as a cell surface marker, which may be used to confirm transduction or engineering of the cell to express the receptor, such as a truncated version of a cell surface receptor, such as truncated EGFR (tEGFR). In some aspects, the marker includes all or part (e.g., truncated form) of CD34, a NGFR, or epidermal growth factor receptor (e.g., tEGFR). In some embodiments, the nucleic acid encoding the marker is operably linked to a polynucleotide encoding for a linker sequence, such as a cleavable linker sequence, e.g., T2A. For example, a marker, and optionally a linker sequence, can be any as disclosed in published patent application No. WO2014031687. For example, the marker can be a truncated EGFR (tEGFR) that is, optionally, linked to a linker sequence, such as a T2A cleavable linker sequence. An exemplary polypeptide for a truncated EGFR (e.g. tEGFR) comprises the sequence of amino acids set forth in SEQ ID NO: 111 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 111. An exemplary T2A linker sequence comprises the sequence of amino acids set forth in SEQ ID NO: 110 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 110.

[0114] In some embodiments, the marker is a molecule, e.g., cell surface protein, not naturally found on T cells or not naturally found on the surface of T cells, or a portion thereof.

[0115] In some embodiments, the molecule is a non-self molecule, e.g., non-self protein, i.e., one that is not recognized as “self” by the immune system of the host into which the cells will be adoptively transferred.

[0116] In some embodiments, the marker serves no therapeutic function and/or produces no effect other than to be used as a marker for genetic engineering, e.g., for selecting cells successfully engineered. In other embodiments, the marker may be a therapeutic molecule or molecule otherwise exerting some desired effect, such as a ligand for a cell to be encountered *in vivo*, such as a costimulatory or immune checkpoint molecule to enhance and/or dampen responses of the cells upon adoptive transfer and encounter with ligand.

[0117] In some cases, CARs are referred to as first, second, and/or third generation CARs. In some aspects, a first generation CAR is one that solely provides a CD3-chain induced signal upon antigen binding; in some aspects, a second-generation CARs is one that provides such a signal and costimulatory signal, such as one including an intracellular signaling domain from a

costimulatory receptor such as CD28 or CD137; in some aspects, a third generation CAR is one that includes multiple costimulatory domains of different costimulatory receptors.

[0118] In some embodiments, the chimeric antigen receptor includes an extracellular portion containing an antibody or antibody fragment. In some aspects, the chimeric antigen receptor includes an extracellular portion containing the antibody or fragment and an intracellular signaling domain. In some embodiments, the antibody or fragment includes an scFv and the intracellular domain contains an ITAM. In some aspects, the intracellular signaling domain includes a signaling domain of a zeta chain of a CD3-zeta (CD3 ζ) chain. In some embodiments, the chimeric antigen receptor includes a transmembrane domain linking the extracellular domain and the intracellular signaling domain. In some aspects, the transmembrane domain contains a transmembrane portion of CD28. In some embodiments, the transmembrane domain of the receptor, e.g., the CAR is a transmembrane domain of human CD28 or variant thereof, e.g., a 27-amino acid transmembrane domain of a human CD28 (Accession No.: P10747.1). In some embodiments, the chimeric antigen receptor contains an intracellular domain of a T cell costimulatory molecule. The extracellular domain and transmembrane domain can be linked directly or indirectly. In some embodiments, the extracellular domain and transmembrane are linked by a spacer, such as any described herein. In some embodiments, the receptor contains extracellular portion of the molecule from which the transmembrane domain is derived, such as a CD28 extracellular portion. In some embodiments, the chimeric antigen receptor contains an intracellular domain derived from a T cell costimulatory molecule or a functional variant thereof, such as between the transmembrane domain and intracellular signaling domain. In some aspects, the T cell costimulatory molecule is CD28 or 41BB.

[0119] For example, in some embodiments, the CAR contains an antibody, e.g., an antibody fragment, a transmembrane domain that is or contains a transmembrane portion of CD28 or a functional variant thereof, and an intracellular signaling domain containing a signaling portion of CD28 or functional variant thereof and a signaling portion of CD3 zeta or functional variant thereof. In some embodiments, the CAR contains an antibody, e.g., antibody fragment, a transmembrane domain that is or contains a transmembrane portion of CD28 or a functional variant thereof, and an intracellular signaling domain containing a signaling portion of a 4-1BB or functional variant thereof and a signaling portion of CD3 zeta or functional variant thereof. In some such embodiments, the receptor further includes a spacer containing a portion of an Ig

molecule, such as a human Ig molecule, such as an Ig hinge, e.g. an IgG4 hinge, such as a hinge-only spacer.

[0120] In some embodiments, the transmembrane domain of the recombinant receptor, e.g., the CAR, is or includes a transmembrane domain of human CD28 (e.g. Accession No. P01747.1) or variant thereof, such as a transmembrane domain that comprises the sequence of amino acids set forth in SEQ ID NO: 2 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 2; in some embodiments, the transmembrane-domain containing portion of the recombinant receptor comprises the sequence of amino acids set forth in SEQ ID NO: 104 or a sequence of amino acids having at least at or about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity thereto.

[0121] In some embodiments, the chimeric antigen receptor contains an intracellular domain of a T cell costimulatory molecule. In some aspects, the T cell costimulatory molecule is CD28 or 41BB.

[0122] In some embodiments, the intracellular signaling component(s) of the recombinant receptor, e.g. the CAR, contains an intracellular costimulatory signaling domain of human CD28 or a functional variant or portion thereof, such as a domain with an LL to GG substitution at positions 186-187 of a native CD28 protein. For example, the intracellular signaling domain can comprise the sequence of amino acids set forth in SEQ ID NO: 112 or 113 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 112 or 113. In some embodiments, the intracellular domain comprises an intracellular costimulatory signaling domain of 4-1BB (e.g. (Accession No. Q07011.1) or functional variant or portion thereof, such as the sequence of amino acids set forth in SEQ ID NO: 3 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 3.

[0123] In some embodiments, the intracellular signaling domain of the recombinant receptor, e.g. the CAR, comprises a human CD3 zeta stimulatory signaling domain or functional variant thereof, such as an 112 AA cytoplasmic domain of isoform 3 of human CD3 ζ (Accession No.: P20963.2) or a CD3 zeta signaling domain as described in U.S. Patent No.: 7,446,190 or U.S. Patent No. 8,911,993. For example, in some embodiments, the intracellular signaling

domain comprises the sequence of amino acids as set forth in SEQ ID NO: 4, 105 or 159 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 4, 105 or 159.

[0124] In some aspects, the spacer contains only a hinge region of an IgG, such as only a hinge of IgG4 or IgG1, such as the hinge only spacer set forth in SEQ ID NO: 1. In other embodiments, the spacer is or contains an Ig hinge, e.g., an IgG4-derived hinge, optionally linked to a CH2 and/or CH3 domains. In some embodiments, the spacer is an Ig hinge, e.g., an IgG4 hinge, linked to CH2 and CH3 domains, such as set forth in SEQ ID NO: 108. In some embodiments, the spacer is an Ig hinge, e.g., an IgG4 hinge, linked to a CH3 domain only, such as set forth in SEQ ID NO: 107. In some embodiments, the spacer is or comprises a glycine-serine rich sequence or other flexible linker such as known flexible linkers.

[0125] For example, in some embodiments, the CAR includes an antibody such as an antibody fragment, including scFvs, a spacer, such as a spacer containing a portion of an immunoglobulin molecule, such as a hinge region and/or one or more constant regions of a heavy chain molecule, such as an Ig-hinge containing spacer, a transmembrane domain containing all or a portion of a CD28-derived transmembrane domain, a CD28-derived intracellular signaling domain, and a CD3 zeta signaling domain. In some embodiments, the CAR includes: an extracellular ligand-binding portion, such as an antigen-binding portion, such as an antibody or fragment thereof, including sdAbs and scFvs, that specifically binds an antigen, e.g. an antigen described herein; a spacer such as any of the Ig-hinge containing spacers; a transmembrane domain that is a portion of CD28 or a variant thereof; an intracellular signaling domain containing a signaling portion of 4-1BB or functional variant thereof; and a signaling portion of CD3 zeta signaling domain or functional variant thereof.

[0126] In some embodiments, nucleic acid molecules encoding such CAR constructs further includes a sequence encoding a T2A ribosomal skip element and/or a tEGFR sequence, e.g., downstream of the sequence encoding the CAR. In some embodiments, the sequence encodes a T2A ribosomal skip element and/or a tEGFR sequence set forth in SEQ ID NO: 110 and/or 111, respectively, or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO: 110 and/or 111. In some embodiments, T cells expressing an antigen receptor (e.g. CAR) can also be generated to express a truncated EGFR (EGFRt) as a non-immunogenic selection epitope (e.g. by introduction of a construct encoding the CAR and EGFRt separated by a T2A

ribosome switch to express two proteins from the same construct), which then can be used as a marker to detect such cells (see e.g. U.S. Patent No. 8,802,374).

2. *Exemplary Modifications of a Chimeric Receptor*

[0127] In some embodiments, the variant chimeric receptor contains a modified junction region containing one or more modifications (e.g. insertions, deletions or replacements) in a junction region of a reference chimeric receptor as described, wherein the reference chimeric receptor contains a first domain that is or comprises an extracellular ligand binding domain or a portion thereof and a second domain that is or comprises a hinge domain or a portion thereof, joined in contiguous sequence at a junction.

[0128] In some embodiments, the variant chimeric receptor contains a modified junction region containing one or more modifications (e.g. insertions, deletions or replacements) in a junction region of a reference chimeric receptor as described, wherein the reference chimeric receptor contains a first domain that is or comprises a hinge domain or a portion thereof and a second domain that is or comprises a transmembrane domain or a portion thereof, joined in contiguous sequence at a junction.

[0129] In some embodiments, the variant chimeric receptor contains a modified junction region containing one or more modifications (e.g. insertions, deletions or replacements) in a junction region of a reference chimeric receptor as described, wherein the reference chimeric receptor contains a first domain that is or comprises a transmembrane domain or a portion thereof and a second domain that is or comprises a costimulatory signaling domain or a portion thereof, joined in contiguous sequence at a junction.

[0130] In some embodiments, the variant chimeric receptor contains a modified junction region containing one or more modifications (e.g. insertions, deletions or replacements) in a junction region of a reference chimeric receptor as described, wherein the reference chimeric receptor contains a first domain that is or comprises a costimulatory signaling domain or a portion thereof and a second domain that is or comprises an activating cytoplasmic signaling domain or a portion thereof, joined in contiguous sequence at a junction.

[0131] In some embodiments, the first domain of the reference chimeric receptor is or comprises a transmembrane domain or a portion thereof. In some embodiments, the transmembrane domain include those derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD

154 and/or transmembrane regions containing functional variants thereof such as those retaining a substantial portion of the structural, e.g., transmembrane, properties thereof. In some embodiments, the transmembrane domain is a transmembrane domain derived from CD4, CD28, or CD8, e.g., CD8alpha, or functional variant thereof. In some embodiments, the transmembrane domain is derived from a human protein or is human. In some embodiments, the second domain of the reference chimeric receptor is or comprises a costimulatory signaling domain, which is directly linked or joined to the transmembrane domain. In some embodiments, the costimulatory signaling domain is or comprises a signaling domain of CD28, 4-1BB, OX40, DAP10, and ICOS. In some embodiments, the costimulatory domain is derived from a human protein or is human.

[0132] In some embodiments, the variant chimeric receptor contains a modified junction region containing one or more modifications (e.g. insertions, deletions or replacements) in a junction region of a reference chimeric receptor as described, wherein the reference chimeric receptor contains a first domain that is or comprises a CD28 transmembrane domain or a portion thereof and a second domain that is or comprises a 4-1BB costimulatory signaling domain or a portion thereof, joined in contiguous sequence at a junction. In some embodiments, the CD28 transmembrane domain is or comprises the sequence of amino acids set forth in SEQ ID NO:2, 103 or 104 or is a functional portion or variant thereof comprising a sequence that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:2, 103 or 104. In some embodiments the 4-1BB signaling domain is or comprises the sequence of amino acids set forth in SEQ ID NO:3 or a functional portion or variant thereof comprising a sequence that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% sequence identity to SEQ ID NO:3. In some embodiments, the first domain and second domain together comprise or have the sequence of amino acids set forth in SEQ ID NO:5 or a functional portion or variant thereof comprising a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:5. In some embodiments, the first domain and second domain of the reference chimeric receptor together have or comprise the sequence of amino acids set forth in SEQ ID NO:5.

[0133] In some embodiments, the variant chimeric receptor comprises a modified junction region that is less than 100% identical in sequence to SEQ ID NO:137 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% identical to SEQ ID NO:137

and includes the modification(s), such as any as described. In some embodiments, the variant chimeric receptor has or comprises a sequence of amino acids that is less than 100% sequence identity to SEQ ID NO:5 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% identical to SEQ ID NO:5 and includes the modification(s), such as any as described. In some embodiments, the variant chimeric receptor has or comprises a sequence of amino acids that is less than 100% identical in sequence to SEQ ID NO:160 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% identical to SEQ ID NO:160 and includes the modification(s), such as any as described. In some embodiments, the variant chimeric receptor has or comprises a modified junction region comprising the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184, a functional variant thereof comprising a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184, or a functional portion thereof, each that include the modification(s), such as any as described.

[0134] In some embodiments, the variant chimeric receptor does not contain a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, such as the CD28 transmembrane domain. In some embodiments, the variant chimeric receptor contains one or more modifications at or of a hydrophobic amino acid residues or within a hydrophobic portion in the transmembrane domain, such as the CD28 transmembrane domain. In some embodiments, the one or more modifications is or comprises a substitution of the hydrophobic amino acid with another different hydrophobic amino acid residue. In some embodiments, the one or more modifications is not or does not comprise a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain other than a substitution with another hydrophobic amino acid residue.

[0135] In some cases, transmembrane domains contain one or more tryptophan residues that interact with the lipid bilayers of a membrane. In some cases, the one or more tryptophan residues can be located near the lipid-water interface. In some cases, the one or more tryptophan residues anchor or assist in anchoring the transmembrane domain within the membrane. *See e.g.* de Jesus and Allen, *Biochim Biophys Acta*. 2013 Feb;1828(2):864-76. In some embodiments, the variant chimeric receptor does not contain a modification at one or both of a tryptophan residue in the transmembrane domain, such as the CD28 transmembrane domain. In some embodiments, the chimeric receptor does not contain a modification at an amino acid position

corresponding to position 2 and/or position 26 with reference to numbering of SEQ ID NO: 5, each of which corresponds to a tryptophan in the reference chimeric receptor.

[0136] In some aspects, the hydrophobic character of a chimeric receptor, or a portion thereof, is assessed. In some embodiments, programs such as grand average of hydropathy (GRAVY) are used to assess global hydrophobicity of the chimeric receptor (e.g. variant chimeric receptor), or a portion thereof. In some embodiments, programs are used such as those available via ExPASy (Switzerland) to determine the global hydrophobicity score for a chimeric receptor, or portion thereof. In some aspects, the chimeric receptor comprises a transmembrane domain, which may have a hydrophobicity score indicating that the global character of this domain is hydrophobic. In some aspects, the chimeric receptor comprises one or more domains other than a transmembrane domain, which may have hydrophobicity scores indicating that the one or more other domains are globally hydrophilic. In some aspects, the hydrophobicity for each individual amino acid residue of a given protein sequence (e.g. chimeric receptor or portion thereof) is calculated based upon a hydrophobicity scale. Exemplary amino acid hydrophobicity scales include, but are not limited to, those calculated and/or reported by Kyte and Doolittle (1982), Engelman et al. (1986), Nozaki and Tanford (1971), Miyazawa and Jernigan (1996), Miyazawa and Jernigan (1999), and Black and Mould (1991). In some embodiments, hydrophobicity scales are reported or normalized such that hydrophilic residues, or peptides, are accorded a score below 0 and hydrophobic residues, or peptides, are accorded a score above 0. Thus, in some aspects, a transmembrane domain may have a global hydrophobicity score above 0, or may be comprised of individual residues with hydrophobicity scores above 0. In some embodiments, mutations or modifications to a residue, peptide, chimeric receptor, or portion thereof, are conservative with regard to the unmodified residue or peptide's hydrophobicity score.

[0137] In some embodiments, the variant chimeric receptor or a portion thereof, e.g. a portion containing the modified junction region and/or containing a domain in the variant chimeric receptor that corresponds to the transmembrane domain of the reference chimeric receptor, has a substantially hydrophobic hydropathy profile and/or has a positive grand average of hydropathy (GRAVY) value. In some embodiments, the GRAVY value is greater than 0, 0.25, 0.5, 0.75, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 or greater.

[0138] In some embodiments, the variant chimeric receptor does not contain a modification at or of an amino acid residue involved in or necessary for the signaling of the costimulatory signaling domain, such as at or of an amino acid residue involved in or necessary for 4-1BB signaling. In general, costimulatory signaling involves interactions with TRAF molecules. In some embodiments, the variant chimeric receptor does not contain a modification at or of an amino acid residue that interacts with or is part of a binding motif for binding to a TRAF molecule. In some embodiments, the variant chimeric receptor does not contain a modification at or of an amino acid residue in the costimulatory signaling domain of the reference chimeric receptor that comprises the motif (P/S/A/T)X(Q/E)E. In some embodiments, the TRAF molecule is TRAF 1, TRAF2 and/or TRAF3. In some embodiments, the domain in the variant chimeric receptor that corresponds to the costimulatory signaling domain of the reference chimeric receptor is capable of inducing the activation or cellular localization of a TRAF and/or is capable of inducing TRAF-mediated signaling. In some embodiments, the variant chimeric receptor contains amino acids TTQE at positions corresponding to 49-52 and/or amino acids PEEE at positions corresponding to residues 60-63, each with reference to numbering set forth in SEQ ID NO:5.

[0139] In some embodiments, the variant chimeric receptor contains one or more amino acid modification within a portion between residue 13 and 42 or between amino acid residue 15 and 40, with reference to numbering set forth in SEQ ID NO:5.

[0140] In some embodiments, the modification is or includes insertion of one or more amino acid residues. In some embodiments, the one or more insertion is between amino acid residues adjacent to the junction between the domains. In some embodiments, the one or more amino acid insertions is between amino acid residues 27 and 28 with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the one or more insertions can include insertion of up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues. In some embodiments, the insertion is of 1, 2, 3, 4, or 5 amino acid residues. In some embodiments, the insertion is to any amino acid residues. In some embodiments, the insertion of is insertion of an asparagine (N).

[0141] In some embodiments, the modification is or includes one or more amino acid replacements at a residue corresponding to residue 28, 31 or 34 with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the amino acid replacement can be to any other amino acid. In some embodiments, the amino acid replacement is to an amino acid residue that is leucine (L), asparagine (N), glutamine (Q), alanine (A), serine (S) or histidine (H). In some

embodiments, the amino acid replacement is or corresponds to one or more of K28A, K28H, K28L, K28Q, K28S, R31A, R31H, R31L, R31N, R31S, L34A and L34S, with reference to numbering set forth in SEQ ID NO:5.

[0142] In some embodiments, the variant chimeric receptor contains a modified junction region with two or more, such as up to 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid modifications compared to a junction region of a reference chimeric receptor. In some embodiments, the amino acid modification includes amino acid replacements corresponding to two or more of K28A, K28H, K28L, K28Q, K28S, R31A, R31H, R31L, R31N, R31S, L34A and L34S, with reference to numbering set forth in SEQ ID NO:5. In some embodiments, the amino acid replacements are or correspond to amino acid replacements selected from among K28Q/R31A, K28Q/R31N, K28Q/R31S, K28Q/L34A, K28Q/L34S, R31N/L34A, R31N/L34S, K28Q/R31N/L34A, K28Q/R31N/L34S.

[0143] In some embodiments, the variant chimeric receptor has or comprises a modified junction region that has the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184, or a functional variant thereof comprising a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184, or a functional portion thereof, each that includes the modification(s).

[0144] In some embodiments, the variant chimeric receptor has or comprises the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183, a functional variant thereof comprising a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183, or a functional portion thereof, each that includes the modification(s).

3. Features of Variant Chimeric Receptors

[0145] In some embodiments, the variant chimeric receptor contains a modified junction region such that peptide fragments of or derived from such region exhibit a lower binding affinity for a human leukocyte antigen (HLA) and/or the region exhibits reduced immunogenicity, including following administration to a subject.

[0146] In some embodiments, a peptide fragment having the sequence of an 8-15 amino acid portion of the modified junction region has a binding affinity for a human leukocyte antigen (HLA) molecule that is lower than the binding affinity, for the same HLA molecule, of a peptide

fragment having the sequence of the corresponding portion of the junction region of the reference chimeric receptor. In some embodiments, the peptide fragment of the corresponding portion of the junction region of the reference chimeric receptor has a binding affinity of less than 1000 nM, less than 500 nM or less than 50 nM.

[0147] In some embodiments, the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the modified junction region for a human HLA molecule is lower than the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the junction region of the reference chimeric receptor. In some embodiments, the binding affinity or average of binding affinities is more than 2-fold, more than 5-fold, more than 10-fold, more than 25-fold, more than 50-fold or more than 100-fold lower.

[0148] In some embodiments, the number of peptide fragments having the sequence of an 8-15 amino acid portion of the modified junction region that has a binding affinity for a human leukocyte antigen (HLA) of less than 1000 nM is reduced compared to the number of peptide fragments having the sequence of an 8-15 amino acid portion of the junction region of the reference chimeric receptor that has the same affinity for binding the same HLA. In some embodiments, the number of peptide fragments having the sequence of an 8-15 amino acid portion of the modified junction region that has a binding affinity for a human leukocyte antigen (HLA) of less than 500 nM is reduced compared to the number of peptide fragments having the sequence of an 8-15 amino acid portion of the junction region of the reference chimeric receptor that has the same affinity for binding the same HLA. In some embodiments, the number of peptide fragments having the sequence of an 8-15 amino acid portion of the modified junction region that has a binding affinity for a human leukocyte antigen (HLA) of less than 50 nM is reduced compared to the number of peptide fragments having the sequence of an 8-15 amino acid portion of the junction region of the reference chimeric receptor that has the same affinity for binding the same HLA.

[0149] In some embodiments, the binding affinity can be determined experimentally or algorithmically. In some embodiments, a peptide binding affinity for an MHC can be determined computationally, such as by using algorithms based on quantitative binding affinity models (Lafuente and Reche (2009) *Current Pharmaceutical Design*, 15:3209-3220). In some embodiments, the binding affinity can be determined in an in vitro assay.

[0150] In some embodiments, determining a peptide's binding affinity to an MHC molecule involves radioactivity or fluorescence competition binding assays. See, e.g. Ettinger et al., *J. Immunol.* 160:2365 (1998). In some embodiments, the competition assay yield a comparison of binding affinities of different peptides. Some MHC binding studies utilize detergent solubilized class I molecules from EBV transformed cell lines (see, e.g. Sette, A., et al., *Mol Immunol*, 31(11):813-22 (1994). In some embodiments, the competitive assay involves naturally loaded MHC, and the MHC molecule of interest can be purified away from other MHC molecules in the detergent lysate or be used in a mixture with other MHC molecules. In some embodiments, radiolabeled peptides can be identified that have a high affinity for the MHC molecule in question. In some embodiments, the affinity of additional "test" peptides for the MHC molecule in question is then determined by their ability to compete with the high affinity radiolabeled peptide.

[0151] In some embodiments, determining peptide affinity can involve a reconstitution assay, e.g. using "T2" cells, in which cells expressing an appropriate MHC allele are "stripped" of a native binding peptide by incubating at pH 2-3 for a short period of time. In some embodiments, to determine the binding affinity of a putative MHC-binding peptide for the same MHC allele, the stripped MHC monomer can be combined in solution with the putative MHC-binding peptide, beta2-microglobulin and a conformation-dependent monoclonal antibody. In some embodiments, the difference in fluorescence intensity determined between cells incubated with and without the test binding peptide after labeling, for example, either directly with the labeled monoclonal antibody or a fluorescence-labeled secondary antibody, can be used to determine binding of the test peptide.

[0152] In some embodiments, the binding affinity for an MHC (e.g. HLA) molecule is represented by an IC₅₀, which is the concentration of peptide in a binding assay at which 50% inhibition of binding of a reference peptide is observed. In some cases, such assays can be run under conditions in which IC₅₀ values approximate K_D values (i. e., limiting HLA proteins and labeled peptide concentrations). In some embodiments, binding can be expressed relative to a reference peptide.

[0153] In some embodiments, the binding affinity can be predicted using in silico methods. Exemplary in silico methods for predicting binding affinity for MHC binding using algorithmic or other computational methods are known in the art, See, for example, Marsh, et al., *The HLA Factsbook* (Academic Press, 2000). In some embodiments, an algorithm can be used to predict

if a peptide of interest should bind to a given MHC molecule. See, e.g., Southwood, et al., *J. Immunol.* 160:3363 (1998); Honeyman, et al., *Nat. Biotechnol.* 16:966-969 (1998); Breisie, et al., *Bioinformatics* 14:121-131 (1998), as well as the “SYFPEITHI” algorithm (Hans-Georg Rammensee, et al., *Immunogenetics* (1999) 50: 213-219), Zhang et al., *PLoS ONE* 7(2): e30483. doi: 10.1371/journal.pone.0030483, the Immune Epitope and Analysis Resource (IEDB) (Peters B, et al. *PLoS Biology* 3: 379 (2005)), and the “BIMAS” algorithm (Parker, K. C., M. A. Bednarek, and J. E. Coligan. *J. Immunol.* 152:163 (1994).

[0154] In some embodiments, algorithm prediction tools, including those available from IEDB, use one or more predictions using ANN (Nielsen et al. (2003) *Protein Sci.*, 12:1007-1017 and Lundegaard et al. (2008) *NAR*, 36:W509-512), SMM (Peters and Sette (2005) *BMC Bioinformatics*, 6:132) and comblib (Sidney et al. (2008) *Immunome Res.* 4:2), or the Consensus tool (see Kim, et al. (2012) Immune epitope database analysis resource, *NAR*, combining predictions from any of the foregoing).

[0155] In some embodiments, prediction of antigen processing can be accomplished using an algorithm for proteasomal cleavage (PaProC). See Kuttler et al., *J. Mol. Biol.* 298 (2000), 417-429 and Nussbaum et al., *Immunogenetics* 53 (2001), 87-94.

[0156] In some embodiments, a chimeric receptor (e.g. a CAR), including a variant chimeric receptor (e.g. a variant CAR) can be assessed to determine if a detectable immune response to the molecule is induced following administration to a subject. In some embodiments, a subject can be treated with a chimeric receptor, such as a CAR, for example, by administering cells genetically engineered with the chimeric receptor or CAR. The presence of a humoral or cell-mediated human response can be assessed. In some embodiments, the presence of antibodies that specifically bind to and/or neutralize binding epitopes of the chimeric receptor (e.g. CAR) can be identified by methods such as ELISpot, intracellular cytokine staining, ELISAs (e.g. for cytokines), or cell-based antibody detection methods, for example, by flow cytometry, on serum from the subject. In some embodiments, a cell-mediated immune response to the chimeric receptor, such as a chimeric receptor expressed on engineered cells, can be assessed using a cytotoxic T-lymphocyte (CTL) assay for detection of CD8+ T cells that specifically bind to and induce cytotoxicity and/or a mixed lymphocyte reaction, using cells, e.g., irradiated cells, expressing the chimeric receptor, as stimulator cells.

[0157] In some embodiments, an immune response can be assessed in an in vitro assay by stimulating isolated immune cells (e.g. PBMCs) from a subject administered with a chimeric receptor (e.g. CAR) with a synthetic library of peptides which overlap and span the length of the particular antigen. The immune response in stimulated cells can be assessed using methods that include, but are not limited to, ELISpot, intracellular cytokine staining, cytotoxic T-lymphocyte assays and/or mixed lymphocyte reactions. In some embodiments, T cell activation is measured via T cell proliferation (for example using ³H-thymidine incorporation) or cytokine production. Activation of TH1-type CD4+ T cells can, for example, may be detected via IFN γ production which may be detected by standard techniques, such as an ELISPOT assay.

[0158] In some embodiments, an enzyme-linked immunospot (ELISpot) assay can be employed to assess an immune response to a chimeric receptor. In some aspects, an ELISpot can be adapted for the detection of individual cells secreting specific cytokines or other effector molecules by attachment of a monoclonal antibody specific for a cytokine or effector molecule on a microplate. Cells stimulated by an antigen can be contacted with the immobilized antibody. After washing away cells and any unbound substances, a tagged polyclonal antibody or more often, a monoclonal antibody, specific for the same cytokine or other effector molecule can be added to the wells. Following a wash, a colorant that binds to the tagged antibody can be added such that a blue-black colored precipitate (or spot) forms at the sites of cytokine localization. The spots can be counted manually or with automated ELISpot reader system to quantitated the response.

[0159] In some embodiments, CD8+ T cell-mediated cytotoxic activity can be employed to assess an immune response to the chimeric receptor. In some embodiments, cells stimulated with an antigen (e.g. splenocytes) can be analyzed by chromium release assay for cytotoxicity against ⁵¹Cr-labeled cells. This assay can measure the release of (biological) radioactive chromium from cells as a result of killer cell activity. The cells can be re-stimulated with an antigen in vitro, for example, cells genetically engineered with a chimeric receptor (e.g. CAR-expressing cells), including T cells, peptide-loaded T cells and/or non-transduced ("Mock") T cells (targets) at various effector-to-target (E/T) ratios. Following co-incubation, release of chromium can be quantified and the percentage of maximum achievable lysis in each sample determined. Conditions in which no cytolytic activity specific is detected indicates that a specific immune responses to the chimeric receptor did not develop following co-administration with the tolerogenic peptide. This result may indicate a potential hit for administration of a

specific peptide under conditions capable of inducing immunogenic tolerance to the chimeric receptor.

[0160] In some embodiments, intracellular cytokine staining is employed to assess an immune response to the chimeric receptor, such as in the presence or absence of co-administration with the tolerogenic peptide. Immune cells, such as splenocytes, can be isolated from the mice and stained with antibodies to detect CD8 and CD4 surface expression and intracellular expression of cytokine, including, for example, IFN γ . Conditions in which intracellular cytokine staining indicates T cells did not generate a specific immune response to the chimeric receptor may indicate a potential hit for administration of a specific peptide under conditions capable of inducing immunogenic tolerance to the chimeric receptor.

[0161] In some embodiments, clonal sequencing is employed to assess an immune response to the chimeric receptor. T cell receptors from T cells isolated from challenged mice or patients can be sequenced (see Arstila et al. (1999) Science 286, 958-961; WO 2012/083069). The absence of clonal TCR expansion indicates T cells did not generate a specific immune response to the chimeric receptor. This may indicate a potential hit for administration of a specific peptide under conditions capable of inducing immunogenic tolerance to the chimeric receptor.

[0162] In some embodiments, the variant chimeric receptor exhibits a reduced detectable immune response compared to the immune response generated to the reference chimeric receptor. In some embodiments, the immune response is a humoral immune response. In some embodiments, the immune response is a cell-mediated immune response. In some embodiments, the immune response is reduced greater than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 50-fold, 100-fold or more.

[0163] In some embodiments, the variant chimeric receptor exhibits reduced immunogenicity compared to the reference chimeric receptor (e.g. unmodified or parent chimeric receptor) upon administration to a human subject, optionally wherein the subject has received administration of the reference chimeric receptor. In some embodiments, the reduced immunogenicity comprises a reduced CD4⁺ T cell immune response and/or a reduced CD8⁺ T cell immune response. In some embodiments, immunogenicity is reduced greater than 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 50-fold, 100-fold or more.

[0164] In some embodiments, the maximum number of variant chimeric receptor (e.g. CAR)-expressing cells, the area under the curve (AUC) for variant chimeric receptor-expressing cells over time, and/or the duration of detectable variant chimeric receptor-expressing cells in the subject following the administration of cells expressing the variant chimeric receptor is greater as compared to that achieved via a method comprising administration of cells expressing the reference chimeric receptor. In some embodiments, the method results in a maximum concentration or number of variant chimer receptor-expressing cells in the blood of the subject of at least at or about 10 chimeric-expressing cells per microliter, at least 50 % of the total number of peripheral blood mononuclear cells (PBMCs), at least at least about 1×10^5 chimeric receptor-expressing cells, or at least 1,000, or at least 2,000, or at least 3,000, or at least 4,000, or at least 5,000 copies of CAR-encoding DNA per micrograms DNA. In some embodiments, at day 30, at day 60, or at day 90 following the initiation of the administration of cells expressing the variant chimer receptor, variant chimeric receptor -expressing cells are detectable in the blood or serum of the subject.

[0165] In some embodiments, the variant chimeric receptor exhibits reduced immunogenicity as described while also retaining the biological activity or function, such as cytotoxic activity, as compared to the reference chimeric receptor (unmodified or parent chimeric receptor not containing the amino acid replacement(s)). In some embodiments, the biologic activity, such as cytotoxic activity, is present in an antigen-specific manner. In some embodiments, the biological or functional activity, such as cytotoxic activity, of the variant chimeric receptor is at least 40%, 50%, 60%, 70%, such as typically at least 75%, 80%, 85%, 90%, 95% of the biological activity or functional activity (e.g. cytotoxic activity) of the unmodified or parent chimeric receptor not containing the amino acid replacement(s).

[0166] In some embodiments, biological activity or functional activity of a chimeric receptor, such as cytotoxic activity, can be measured using any of a number of known methods. The activity can be assessed or determined either in vitro or in vivo. In some embodiments, activity can be assessed once the cells are administered to the subject (e.g., human). Parameters to assess include specific binding of an engineered or natural T cell or other immune cell to antigen, e.g., in vivo, e.g., by imaging, or ex vivo, e.g., by ELISA or flow cytometry. In certain embodiments, the ability of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer et al., *J. Immunotherapy*, 32(7): 689-702 (2009), and Herman et al. *J.*

Immunological Methods, 285(1): 25-40 (2004). In certain embodiments, the biological activity of the cells also can be measured by assaying expression and/or secretion of certain cytokines, such as CD 107a, IFN γ , IL-2, and TNF. In some aspects the biological activity is measured by assessing clinical outcome, such as reduction in tumor burden or load.

III. Nucleic Acids, Vectors and Engineered Cells

[0167] Provided are methods, nucleic acids, compositions, and kits for producing the genetically engineered cells. The genetic engineering generally involves introduction of a nucleic acid encoding the chimeric receptor into a composition containing the cultured cells, such as by retroviral transduction, transfection, or transformation.

[0168] In some embodiments, the nucleic acid molecule encodes the recombinant receptors, e.g., chimeric receptor, such as any described above. Also provided are vectors or constructs containing such nucleic acid molecules. In some embodiments, the vectors or constructs contain one or more promoters operatively linked to the nucleotide encoding the receptor to drive expression thereof. In some embodiments, the promoter is operatively linked to one or more than one nucleic acid molecule.

[0169] In some embodiments, the vector or construct can contain a single promoter that drives the expression of one or more nucleic acid molecules. In some embodiments, such promoters can be multicistronic (bicistronic or tricistronic, see e.g., U.S. Patent No. 6,060,273). For example, in some embodiments, transcription units can be engineered as a bicistronic unit containing an IRES (internal ribosome entry site), which allows coexpression of gene products (e.g. encoding a first and second chimeric receptor) by a message from a single promoter. Alternatively, in some cases, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three genes (e.g. encoding a first and second chimeric receptor) separated from one another by sequences encoding a self-cleavage peptide (e.g., T2A) or a protease recognition site (e.g., furin). The ORF thus encodes a single polyprotein, which, either during (in the case of T2A) or after translation, is cleaved into the individual proteins. In some cases, the peptide, such as T2A, can cause the ribosome to skip (ribosome skipping) synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream. Examples of 2A cleavage peptides, including those that can induce ribosome skipping, are T2A, P2A, E2A and F2A.

[0170] Also provided are cells such as cells that contain an engineered chimeric receptor, such as described herein. Also provided are populations of such cells, compositions containing such cells and/or enriched for such cells, such as in which cells expressing the chimeric receptor make up at least 50, 60, 70, 80, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or more percent of the total cells in the composition or cells of a certain type such as T cells or CD8+ or CD4+ cells. Among the compositions are pharmaceutical compositions and formulations for administration, such as for adoptive cell therapy. Also provided are therapeutic methods for administering the cells and compositions to subjects, e.g., patients.

[0171] Thus also provided are genetically engineered cells expressing the chimeric receptors e.g., cells containing the CARs. The cells generally are eukaryotic cells, such as mammalian cells, and typically are human cells. In some embodiments, the cells are derived from the blood, bone marrow, lymph, or lymphoid organs, are cells of the immune system, such as cells of the innate or adaptive immunity, e.g., myeloid or lymphoid cells, including lymphocytes, typically T cells and/or NK cells. Other exemplary cells include stem cells, such as multipotent and pluripotent stem cells, including induced pluripotent stem cells (iPSCs). The cells typically are primary cells, such as those isolated directly from a subject and/or isolated from a subject and frozen. In some embodiments, the cells include one or more subsets of T cells or other cell types, such as whole T cell populations, CD4+ cells, CD8+ cells, and subpopulations thereof, such as those defined by function, activation state, maturity, potential for differentiation, expansion, recirculation, localization, and/or persistence capacities, antigen-specificity, type of antigen receptor, presence in a particular organ or compartment, marker or cytokine secretion profile, and/or degree of differentiation. With reference to the subject to be treated, the cells may be allogeneic and/or autologous. Among the methods include off-the-shelf methods. In some aspects, such as for off-the-shelf technologies, the cells are pluripotent and/or multipotent, such as stem cells, such as induced pluripotent stem cells (iPSCs). In some embodiments, the methods include isolating cells from the subject, preparing, processing, culturing, and/or engineering them, as described herein, and re-introducing them into the same patient, before or after cryopreservation.

[0172] Among the sub-types and subpopulations of T cells and/or of CD4+ and/or of CD8+ T cells are naïve T (T_N) cells, effector T cells (T_{EFF}), memory T cells and sub-types thereof, such as stem cell memory T (T_{SCM}), central memory T (T_{CM}), effector memory T (T_{EM}), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells,

mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, alpha/beta T cells, and delta/gamma T cells.

[0173] In some embodiments, the cells are natural killer (NK) cells. In some embodiments, the cells are monocytes or granulocytes, e.g., myeloid cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and/or basophils.

[0174] In some embodiments, the cells include one or more nucleic acids introduced via genetic engineering, and thereby express recombinant or genetically engineered products of such nucleic acids. In some embodiments, the nucleic acids are heterologous, i.e., normally not present in a cell or sample obtained from the cell, such as one obtained from another organism or cell, which for example, is not ordinarily found in the cell being engineered and/or an organism from which such cell is derived. In some embodiments, the nucleic acids are not naturally occurring, such as a nucleic acid not found in nature, including one comprising chimeric combinations of nucleic acids encoding various domains from multiple different cell types.

Preparation of cells for engineering

[0175] In some embodiments, preparation of the engineered cells includes one or more culture and/or preparation steps. The cells for introduction of the chimeric receptor, e.g., CAR, may be isolated from a sample, such as a biological sample, e.g., one obtained from or derived from a subject. In some embodiments, the subject from which the cell is isolated is one having the disease or condition or in need of a cell therapy or to which cell therapy will be administered. The subject in some embodiments is a human in need of a particular therapeutic intervention, such as the adoptive cell therapy for which cells are being isolated, processed, and/or engineered.

[0176] Accordingly, the cells in some embodiments are primary cells, e.g., primary human cells. The samples include tissue, fluid, and other samples taken directly from the subject, as well as samples resulting from one or more processing steps, such as separation, centrifugation, genetic engineering (e.g. transduction with viral vector), washing, and/or incubation. The biological sample can be a sample obtained directly from a biological source or a sample that is processed. Biological samples include, but are not limited to, body fluids, such as blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat, tissue and organ samples, including processed samples derived therefrom.

[0177] In some aspects, the sample from which the cells are derived or isolated is blood or a blood-derived sample, or is or is derived from an apheresis or leukapheresis product. Exemplary samples include whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus, tissue biopsy, tumor, leukemia, lymphoma, lymph node, gut associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, liver, lung, stomach, intestine, colon, kidney, pancreas, breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. Samples include, in the context of cell therapy, e.g., adoptive cell therapy, samples from autologous and allogeneic sources.

[0178] In some embodiments, the cells are derived from cell lines, e.g., T cell lines. The cells in some embodiments are obtained from a xenogeneic source, for example, from mouse, rat, non-human primate, or pig.

[0179] In some embodiments, isolation of the cells includes one or more preparation and/or non-affinity based cell separation steps. In some examples, cells are washed, centrifuged, and/or incubated in the presence of one or more reagents, for example, to remove unwanted components, enrich for desired components, lyse or remove cells sensitive to particular reagents. In some examples, cells are separated based on one or more property, such as density, adherent properties, size, sensitivity and/or resistance to particular components.

[0180] In some examples, cells from the circulating blood of a subject are obtained, e.g., by apheresis or leukapheresis. The samples, in some aspects, contain lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and/or platelets, and in some aspects contains cells other than red blood cells and platelets.

[0181] In some embodiments, the blood cells collected from the subject are washed, e.g., to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In some embodiments, the cells are washed with phosphate buffered saline (PBS). In some embodiments, the wash solution lacks calcium and/or magnesium and/or many or all divalent cations. In some aspects, a washing step is accomplished a semi-automated “flow-through” centrifuge (for example, the Cobe 2991 cell processor, Baxter) according to the manufacturer's instructions. In some aspects, a washing step is accomplished by tangential flow filtration (TFF) according to the manufacturer's instructions. In some embodiments, the cells are resuspended in a variety of biocompatible buffers after washing, such as, for example, $\text{Ca}^{++}/\text{Mg}^{++}$ free PBS. In certain embodiments, components of a blood cell sample are removed and the cells directly resuspended in culture media.

[0182] In some embodiments, the methods include density-based cell separation methods, such as the preparation of white blood cells from peripheral blood by lysing the red blood cells and centrifugation through a Percoll or Ficoll gradient.

[0183] In some embodiments, the isolation methods include the separation of different cell types based on the expression or presence in the cell of one or more specific molecules, such as surface markers, e.g., surface proteins, intracellular markers, or nucleic acid. In some embodiments, any known method for separation based on such markers may be used. In some embodiments, the separation is affinity- or immunoaffinity-based separation. For example, the isolation in some aspects includes separation of cells and cell populations based on the cells' expression or expression level of one or more markers, typically cell surface markers, for example, by incubation with an antibody or binding partner that specifically binds to such markers, followed generally by washing steps and separation of cells having bound the antibody or binding partner, from those cells having not bound to the antibody or binding partner.

[0184] Such separation steps can be based on positive selection, in which the cells having bound the reagents are retained for further use, and/or negative selection, in which the cells having not bound to the antibody or binding partner are retained. In some examples, both fractions are retained for further use. In some aspects, negative selection can be particularly useful where no antibody is available that specifically identifies a cell type in a heterogeneous population, such that separation is best carried out based on markers expressed by cells other than the desired population.

[0185] The separation need not result in 100% enrichment or removal of a particular cell population or cells expressing a particular marker. For example, positive selection of or enrichment for cells of a particular type, such as those expressing a marker, refers to increasing the number or percentage of such cells, but need not result in a complete absence of cells not expressing the marker. Likewise, negative selection, removal, or depletion of cells of a particular type, such as those expressing a marker, refers to decreasing the number or percentage of such cells, but need not result in a complete removal of all such cells.

[0186] In some examples, multiple rounds of separation steps are carried out, where the positively or negatively selected fraction from one step is subjected to another separation step, such as a subsequent positive or negative selection. In some examples, a single separation step can deplete cells expressing multiple markers simultaneously, such as by incubating cells with a plurality of antibodies or binding partners, each specific for a marker targeted for negative

selection. Likewise, multiple cell types can simultaneously be positively selected by incubating cells with a plurality of antibodies or binding partners expressed on the various cell types.

[0187] For example, in some aspects, specific subpopulations of T cells, such as cells positive or expressing high levels of one or more surface markers, e.g., CD28⁺, CD62L⁺, CCR7⁺, CD27⁺, CD127⁺, CD4⁺, CD8⁺, CD45RA⁺, and/or CD45RO⁺ T cells, are isolated by positive or negative selection techniques.

[0188] For example, CD3⁺, CD28⁺ T cells can be positively selected using CD3/CD28 conjugated magnetic beads (e.g., DYNABEADS® M-450 CD3/CD28 T Cell Expander).

[0189] In some embodiments, isolation is carried out by enrichment for a particular cell population by positive selection, or depletion of a particular cell population, by negative selection. In some embodiments, positive or negative selection is accomplished by incubating cells with one or more antibodies or other binding agent that specifically bind to one or more surface markers expressed or expressed (marker⁺) at a relatively higher level (marker^{high}) on the positively or negatively selected cells, respectively.

[0190] In some embodiments, T cells are separated from a PBMC sample by negative selection of markers expressed on non-T cells, such as B cells, monocytes, or other white blood cells, such as CD14. In some aspects, a CD4⁺ or CD8⁺ selection step is used to separate CD4⁺ helper and CD8⁺ cytotoxic T cells. Such CD4⁺ and CD8⁺ populations can be further sorted into sub-populations by positive or negative selection for markers expressed or expressed to a relatively higher degree on one or more naive, memory, and/or effector T cell subpopulations.

[0191] In some embodiments, CD8⁺ cells are further enriched for or depleted of naive, central memory, effector memory, and/or central memory stem cells, such as by positive or negative selection based on surface antigens associated with the respective subpopulation. In some embodiments, enrichment for central memory T (T_{CM}) cells is carried out to increase efficacy, such as to improve long-term survival, expansion, and/or engraftment following administration, which in some aspects is particularly robust in such sub-populations. *See* Terakura et al. (2012) *Blood*.1:72–82; Wang et al. (2012) *J Immunother.* 35(9):689-701. In some embodiments, combining T_{CM}-enriched CD8⁺ T cells and CD4⁺ T cells further enhances efficacy.

[0192] In embodiments, memory T cells are present in both CD62L⁺ and CD62L⁻ subsets of CD8⁺ peripheral blood lymphocytes. PBMC can be enriched for or depleted of CD62L⁻CD8⁺ and/or CD62L⁺CD8⁺ fractions, such as using anti-CD8 and anti-CD62L antibodies.

[0193] In some embodiments, the enrichment for central memory T (T_{CM}) cells is based on positive or high surface expression of CD45RO, CD62L, CCR7, CD28, CD3, and/or CD 127; in some aspects, it is based on negative selection for cells expressing or highly expressing CD45RA and/or granzyme B. In some aspects, isolation of a $CD8^+$ population enriched for T_{CM} cells is carried out by depletion of cells expressing CD4, CD14, CD45RA, and positive selection or enrichment for cells expressing CD62L. In one aspect, enrichment for central memory T (T_{CM}) cells is carried out starting with a negative fraction of cells selected based on CD4 expression, which is subjected to a negative selection based on expression of CD14 and CD45RA, and a positive selection based on CD62L. Such selections in some aspects are carried out simultaneously and in other aspects are carried out sequentially, in either order. In some aspects, the same CD4 expression-based selection step used in preparing the $CD8^+$ cell population or subpopulation, also is used to generate the $CD4^+$ cell population or subpopulation, such that both the positive and negative fractions from the CD4-based separation are retained and used in subsequent steps of the methods, optionally following one or more further positive or negative selection steps.

[0194] In a particular example, a sample of PBMCs or other white blood cell sample is subjected to selection of $CD4^+$ cells, where both the negative and positive fractions are retained. The negative fraction then is subjected to negative selection based on expression of CD14 and CD45RA or ROR1, and positive selection based on a marker characteristic of central memory T cells, such as CD62L or CCR7, where the positive and negative selections are carried out in either order.

[0195] $CD4^+$ T helper cells are sorted into naïve, central memory, and effector cells by identifying cell populations that have cell surface antigens. $CD4^+$ lymphocytes can be obtained by standard methods. In some embodiments, naïve $CD4^+$ T lymphocytes are $CD45RO^-$, $CD45RA^+$, $CD62L^+$, $CD4^+$ T cells. In some embodiments, central memory $CD4^+$ cells are $CD62L^+$ and $CD45RO^+$. In some embodiments, effector $CD4^+$ cells are $CD62L^-$ and $CD45RO^-$.

[0196] In one example, to enrich for $CD4^+$ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8. In some embodiments, the antibody or binding partner is bound to a solid support or matrix, such as a magnetic bead or paramagnetic bead, to allow for separation of cells for positive and/or negative selection. For example, in some embodiments, the cells and cell populations are separated or isolated using immunomagnetic (or affinitymagnetic) separation

techniques (reviewed in *Methods in Molecular Medicine*, vol. 58: *Metastasis Research Protocols*, Vol. 2: *Cell Behavior In Vitro and In Vivo*, p 17-25 Edited by: S. A. Brooks and U. Schumacher © Humana Press Inc., Totowa, NJ).

[0197] In some aspects, the sample or composition of cells to be separated is incubated with small, magnetizable or magnetically responsive material, such as magnetically responsive particles or microparticles, such as paramagnetic beads (e.g., such as Dynalbeads or MACS beads). The magnetically responsive material, e.g., particle, generally is directly or indirectly attached to a binding partner, e.g., an antibody, that specifically binds to a molecule, e.g., surface marker, present on the cell, cells, or population of cells that it is desired to separate, e.g., that it is desired to negatively or positively select.

[0198] In some embodiments, the magnetic particle or bead comprises a magnetically responsive material bound to a specific binding member, such as an antibody or other binding partner. There are many well-known magnetically responsive materials used in magnetic separation methods. Suitable magnetic particles include those described in Molday, U.S. Pat. No. 4,452,773, and in European Patent Specification EP 452342 B, which are hereby incorporated by reference. Colloidal sized particles, such as those described in Owen U.S. Pat. No. 4,795,698, and Liberti et al., U.S. Pat. No. 5,200,084 are other examples.

[0199] The incubation generally is carried out under conditions whereby the antibodies or binding partners, or molecules, such as secondary antibodies or other reagents, which specifically bind to such antibodies or binding partners, which are attached to the magnetic particle or bead, specifically bind to cell surface molecules if present on cells within the sample.

[0200] In some aspects, the sample is placed in a magnetic field, and those cells having magnetically responsive or magnetizable particles attached thereto will be attracted to the magnet and separated from the unlabeled cells. For positive selection, cells that are attracted to the magnet are retained; for negative selection, cells that are not attracted (unlabeled cells) are retained. In some aspects, a combination of positive and negative selection is performed during the same selection step, where the positive and negative fractions are retained and further processed or subject to further separation steps.

[0201] In certain embodiments, the magnetically responsive particles are coated in primary antibodies or other binding partners, secondary antibodies, lectins, enzymes, or streptavidin. In certain embodiments, the magnetic particles are attached to cells via a coating of primary antibodies specific for one or more markers. In certain embodiments, the cells, rather than the

beads, are labeled with a primary antibody or binding partner, and then cell-type specific secondary antibody- or other binding partner (e.g., streptavidin)-coated magnetic particles, are added. In certain embodiments, streptavidin-coated magnetic particles are used in conjunction with biotinylated primary or secondary antibodies.

[0202] In some embodiments, the magnetically responsive particles are left attached to the cells that are to be subsequently incubated, cultured and/or engineered; in some aspects, the particles are left attached to the cells for administration to a patient. In some embodiments, the magnetizable or magnetically responsive particles are removed from the cells. Methods for removing magnetizable particles from cells are known and include, e.g., the use of competing non-labeled antibodies, magnetizable particles or antibodies conjugated to cleavable linkers, etc. In some embodiments, the magnetizable particles are biodegradable.

[0203] In some embodiments, the affinity-based selection is via magnetic-activated cell sorting (MACS) (Miltenyi Biotech, Auburn, CA). Magnetic Activated Cell Sorting (MACS) systems are capable of high-purity selection of cells having magnetized particles attached thereto. In certain embodiments, MACS operates in a mode wherein the non-target and target species are sequentially eluted after the application of the external magnetic field. That is, the cells attached to magnetized particles are held in place while the unattached species are eluted. Then, after this first elution step is completed, the species that were trapped in the magnetic field and were prevented from being eluted are freed in some manner such that they can be eluted and recovered. In certain embodiments, the non-target cells are labelled and depleted from the heterogeneous population of cells.

[0204] In certain embodiments, the isolation or separation is carried out using a system, device, or apparatus that carries out one or more of the isolation, cell preparation, separation, processing, incubation, culture, and/or formulation steps of the methods. In some aspects, the system is used to carry out each of these steps in a closed or sterile environment, for example, to minimize error, user handling and/or contamination. In one example, the system is a system as described in International Patent Application, Publication Number WO2009/072003, or US 20110003380 A1.

[0205] In some embodiments, the system or apparatus carries out one or more, e.g., all, of the isolation, processing, engineering, and formulation steps in an integrated or self-contained system, and/or in an automated or programmable fashion. In some aspects, the system or apparatus includes a computer and/or computer program in communication with the system or

apparatus, which allows a user to program, control, assess the outcome of, and/or adjust various aspects of the processing, isolation, engineering, and formulation steps.

[0206] In some aspects, the separation and/or other steps is carried out using CliniMACS system (Miltenyi Biotec), for example, for automated separation of cells on a clinical-scale level in a closed and sterile system. Components can include an integrated microcomputer, magnetic separation unit, peristaltic pump, and various pinch valves. The integrated computer in some aspects controls all components of the instrument and directs the system to perform repeated procedures in a standardized sequence. The magnetic separation unit in some aspects includes a movable permanent magnet and a holder for the selection column. The peristaltic pump controls the flow rate throughout the tubing set and, together with the pinch valves, ensures the controlled flow of buffer through the system and continual suspension of cells.

[0207] The CliniMACS system in some aspects uses antibody-coupled magnetizable particles that are supplied in a sterile, non-pyrogenic solution. In some embodiments, after labelling of cells with magnetic particles the cells are washed to remove excess particles. A cell preparation bag is then connected to the tubing set, which in turn is connected to a bag containing buffer and a cell collection bag. The tubing set consists of pre-assembled sterile tubing, including a pre-column and a separation column, and are for single use only. After initiation of the separation program, the system automatically applies the cell sample onto the separation column. Labelled cells are retained within the column, while unlabeled cells are removed by a series of washing steps. In some embodiments, the cell populations for use with the methods described herein are unlabeled and are not retained in the column. In some embodiments, the cell populations for use with the methods described herein are labeled and are retained in the column. In some embodiments, the cell populations for use with the methods described herein are eluted from the column after removal of the magnetic field, and are collected within the cell collection bag.

[0208] In certain embodiments, separation and/or other steps are carried out using the CliniMACS Prodigy system (Miltenyi Biotec). The CliniMACS Prodigy system in some aspects is equipped with a cell processing unit that permits automated washing and fractionation of cells by centrifugation. The CliniMACS Prodigy system can also include an onboard camera and image recognition software that determines the optimal cell fractionation endpoint by discerning the macroscopic layers of the source cell product. For example, peripheral blood may be automatically separated into erythrocytes, white blood cells and plasma

layers. The CliniMACS Prodigy system can also include an integrated cell cultivation chamber which accomplishes cell culture protocols such as, e.g., cell differentiation and expansion, antigen loading, and long-term cell culture. Input ports can allow for the sterile removal and replenishment of media and cells can be monitored using an integrated microscope. See, e.g., Klebanoff et al. (2012) *J Immunother.* 35(9): 651–660, Terakura et al. (2012) *Blood* 117:72–82, and Wang et al. (2012) *J Immunother.* 35(9):689–701.

[0209] In some embodiments, a cell population described herein is collected and enriched (or depleted) via flow cytometry, in which cells stained for multiple cell surface markers are carried in a fluidic stream. In some embodiments, a cell population described herein is collected and enriched (or depleted) via preparative scale (FACS)-sorting. In certain embodiments, a cell population described herein is collected and enriched (or depleted) by use of microelectromechanical systems (MEMS) chips in combination with a FACS-based detection system (see, e.g., WO 2010/033140, Cho et al. (2010) *Lab Chip* 10, 1567–1573; and Godin et al. (2008) *J Biophoton.* 1(5):355–376. In both cases, cells can be labeled with multiple markers, allowing for the isolation of well-defined T cell subsets at high purity.

[0210] In some embodiments, the antibodies or binding partners are labeled with one or more detectable marker, to facilitate separation for positive and/or negative selection. For example, separation may be based on binding to fluorescently labeled antibodies. In some examples, separation of cells based on binding of antibodies or other binding partners specific for one or more cell surface markers are carried in a fluidic stream, such as by fluorescence-activated cell sorting (FACS), including preparative scale (FACS) and/or microelectromechanical systems (MEMS) chips, e.g., in combination with a flow-cytometric detection system. Such methods allow for positive and negative selection based on multiple markers simultaneously.

[0211] In some embodiments, the preparation methods include steps for freezing, e.g., cryopreserving, the cells, either before or after isolation, incubation, and/or engineering. In some embodiments, the freeze and subsequent thaw step removes granulocytes and, to some extent, monocytes in the cell population. In some embodiments, the cells are suspended in a freezing solution, e.g., following a washing step to remove plasma and platelets. Any of a variety of known freezing solutions and parameters in some aspects may be used. One example involves using PBS containing 20% DMSO and 8% human serum albumin (HSA), or other suitable cell freezing media. This is then diluted 1:1 with media so that the final concentration of

DMSO and HSA are 10% and 4%, respectively. The cells are then frozen to -80°C . at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank.

[0212] In some embodiments, the provided methods include cultivation, incubation, culture, and/or genetic engineering steps. For example, in some embodiments, provided are methods for incubating and/or engineering the depleted cell populations and culture-initiating compositions.

[0213] Thus, in some embodiments, the cell populations are incubated in a culture-initiating composition. The incubation and/or engineering may be carried out in a culture vessel, such as a unit, chamber, well, column, tube, tubing set, valve, vial, culture dish, bag, or other container for culture or cultivating cells.

[0214] In some embodiments, the cells are incubated and/or cultured prior to or in connection with genetic engineering. The incubation steps can include culture, cultivation, stimulation, activation, and/or propagation. In some embodiments, the compositions or cells are incubated in the presence of stimulating conditions or a stimulatory agent. Such conditions include those designed to induce proliferation, expansion, activation, and/or survival of cells in the population, to mimic antigen exposure, and/or to prime the cells for genetic engineering, such as for the introduction of a recombinant antigen receptor.

[0215] The conditions can include one or more of particular media, temperature, oxygen content, carbon dioxide content, time, agents, e.g., nutrients, amino acids, antibiotics, ions, and/or stimulatory factors, such as cytokines, chemokines, antigens, binding partners, fusion proteins, recombinant soluble receptors, and any other agents designed to activate the cells.

[0216] In some embodiments, the stimulating conditions or agents include one or more agent, e.g., ligand, which is capable of activating an intracellular signaling domain of a TCR complex. In some aspects, the agent turns on or initiates TCR/CD3 intracellular signaling cascade in a T cell. Such agents can include antibodies, such as those specific for a TCR component and/or costimulatory receptor, e.g., anti-CD3, anti-CD28, for example, bound to solid support such as a bead, and/or one or more cytokines. Optionally, the expansion method may further comprise the step of adding anti-CD3 and/or anti CD28 antibody to the culture medium (e.g., at a concentration of at least about 0.5 ng/ml). In some embodiments, the stimulating agents include IL-2 and/or IL-15, for example, an IL-2 concentration of at least about 10 units/mL.

[0217] In some aspects, incubation is carried out in accordance with techniques such as those described in US Patent No. 6,040,177 to Riddell et al., Klebanoff et al. (2012) J Immunother. 35(9): 651–660, Terakura et al. (2012) Blood. 117:72–82, and/or Wang et al. (2012) J Immunother. 35(9):689-701.

[0218] In some embodiments, the T cells are expanded by adding to the culture-initiating composition feeder cells, such as non-dividing peripheral blood mononuclear cells (PBMC), (e.g., such that the resulting population of cells contains at least about 5, 10, 20, or 40 or more PBMC feeder cells for each T lymphocyte in the initial population to be expanded); and incubating the culture (e.g. for a time sufficient to expand the numbers of T cells). In some aspects, the non-dividing feeder cells can comprise gamma-irradiated PBMC feeder cells. In some embodiments, the PBMC are irradiated with gamma rays in the range of about 3000 to 3600 rads to prevent cell division. In some aspects, the feeder cells are added to culture medium prior to the addition of the populations of T cells.

[0219] In some embodiments, the stimulating conditions include temperature suitable for the growth of human T lymphocytes, for example, at least about 25 degrees Celsius, generally at least about 30 degrees, and generally at or about 37 degrees Celsius. Optionally, the incubation may further comprise adding non-dividing EBV-transformed lymphoblastoid cells (LCL) as feeder cells. LCL can be irradiated with gamma rays in the range of about 6000 to 10,000 rads. The LCL feeder cells in some aspects is provided in any suitable amount, such as a ratio of LCL feeder cells to initial T lymphocytes of at least about 10:1.

[0220] In embodiments, antigen-specific T cells, such as antigen-specific CD4+ and/or CD8+ T cells, are obtained by stimulating naive or antigen specific T lymphocytes with antigen. For example, antigen-specific T cell lines or clones can be generated to cytomegalovirus antigens by isolating T cells from infected subjects and stimulating the cells in vitro with the same antigen.

Vectors and methods for genetic engineering

[0221] Various methods for the introduction of genetically engineered components, e.g., antigen receptors, e.g., CARs or TCRs, are well known and may be used with the provided methods and compositions. Exemplary methods include those for transfer of nucleic acids encoding the receptors, including via viral vectors, e.g., retroviral or lentiviral, non-viral vectors or transposons, e.g. *Sleeping Beauty* transposon system. Methods of gene transfer can include transduction, electroporation or other method that results into gene transfer into the cell.

[0222] In some embodiments, gene transfer is accomplished by first stimulating the cell, such as by combining it with a stimulus that induces a response such as proliferation, survival, and/or activation, e.g., as measured by expression of a cytokine or activation marker, followed by transduction of the activated cells, and expansion in culture to numbers sufficient for clinical applications.

[0223] In some contexts, overexpression of a stimulatory factor (for example, a lymphokine or a cytokine) may be toxic to a subject. Thus, in some contexts, the engineered cells include gene segments that cause the cells to be susceptible to negative selection in vivo, such as upon administration in adoptive immunotherapy. For example in some aspects, the cells are engineered so that they can be eliminated as a result of a change in the in vivo condition of the patient to which they are administered. The negative selectable phenotype may result from the insertion of a gene that confers sensitivity to an administered agent, for example, a compound. Negative selectable genes include the Herpes simplex virus type I thymidine kinase (HSV-I TK) gene (Wigler et al., Cell 11:223, 1977) which confers ganciclovir sensitivity; the cellular hypoxanthine phosphoribosyltransferase (HPRT) gene, the cellular adenine phosphoribosyltransferase (APRT) gene, bacterial cytosine deaminase, (Mullen et al., Proc. Natl. Acad. Sci. USA. 89:33 (1992)).

[0224] In some embodiments, recombinant nucleic acids are transferred into cells using recombinant infectious virus particles, such as, e.g., vectors derived from simian virus 40 (SV40), adenoviruses, adeno-associated virus (AAV). In some embodiments, recombinant nucleic acids are transferred into T cells using recombinant lentiviral vectors or retroviral vectors, such as gamma-retroviral vectors (see, e.g., Koste et al. (2014) Gene Therapy 2014 Apr 3. doi: 10.1038/gt.2014.25; Carlens et al. (2000) Exp Hematol 28(10): 1137-46; Alonso-Camino et al. (2013) Mol Ther Nucl Acids 2, e93; Park et al., Trends Biotechnol. 2011 November 29(11): 550–557.

[0225] In some embodiments, the retroviral vector has a long terminal repeat sequence (LTR), e.g., a retroviral vector derived from the Moloney murine leukemia virus (MoMLV), myeloproliferative sarcoma virus (MPSV), murine embryonic stem cell virus (MESV), murine stem cell virus (MSCV), spleen focus forming virus (SFFV), or adeno-associated virus (AAV). Most retroviral vectors are derived from murine retroviruses. In some embodiments, the retroviruses include those derived from any avian or mammalian cell source. The retroviruses typically are amphotropic, meaning that they are capable of infecting host cells of several

species, including humans. In one embodiment, the gene to be expressed replaces the retroviral gag, pol and/or env sequences. A number of illustrative retroviral systems have been described (e.g., U.S. Pat. Nos. 5,219,740; 6,207,453; 5,219,740; Miller and Rosman (1989) *BioTechniques* 7:980-990; Miller, A. D. (1990) *Human Gene Therapy* 1:5-14; Scarpa et al. (1991) *Virology* 180:849-852; Burns et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:8033-8037; and Boris-Lawrie and Temin (1993) *Cur. Opin. Genet. Develop.* 3:102-109.

[0226] Methods of lentiviral transduction are known. Exemplary methods are described in, e.g., Wang et al. (2012) *J. Immunother.* 35(9): 689-701; Cooper et al. (2003) *Blood*. 101:1637-1644; Verhoeven et al. (2009) *Methods Mol Biol.* 506: 97-114; and Cavalieri et al. (2003) *Blood*. 102(2): 497-505.

[0227] In some embodiments, recombinant nucleic acids are transferred into T cells via electroporation (see, e.g., Chicaybam et al, (2013) *PLoS ONE* 8(3): e60298 and Van Tedeloo et al. (2000) *Gene Therapy* 7(16): 1431-1437). In some embodiments, recombinant nucleic acids are transferred into T cells via transposition (see, e.g., Manuri et al. (2010) *Hum Gene Ther* 21(4): 427-437; Sharma et al. (2013) *Molec Ther Nucl Acids* 2, e74; and Huang et al. (2009) *Methods Mol Biol* 506: 115-126). Other methods of introducing and expressing genetic material in immune cells include calcium phosphate transfection (e.g., as described in *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.), protoplast fusion, cationic liposome-mediated transfection; tungsten particle-facilitated microparticle bombardment (Johnston, *Nature*, 346: 776-777 (1990)); and strontium phosphate DNA co-precipitation (Brash et al., *Mol. Cell Biol.*, 7: 2031-2034 (1987)).

[0228] Other approaches and vectors for transfer of the nucleic acids encoding the recombinant products are those described, e.g., in international patent application, Publication No.: WO2014055668, and U.S. Patent No. 7,446,190.

[0229] In some embodiments, the cells, e.g., T cells, may be transfected either during or after expansion e.g. with a T cell receptor (TCR) or a chimeric antigen receptor (CAR). This transfection for the introduction of the gene of the desired receptor can be carried out with any suitable retroviral vector, for example. The genetically modified cell population can then be liberated from the initial stimulus (the CD3/CD28 stimulus, for example) and subsequently be stimulated with a second type of stimulus e.g. via a de novo introduced receptor). This second type of stimulus may include an antigenic stimulus in form of a peptide/MHC molecule, the cognate (cross-linking) ligand of the genetically introduced receptor (e.g. natural ligand of a

CAR) or any ligand (such as an antibody) that directly binds within the framework of the new receptor (e.g. by recognizing constant regions within the receptor). See, for example, Cheadle et al., “Chimeric antigen receptors for T-cell based therapy” *Methods Mol Biol.* 2012; 907:645-66 or Barrett et al., *Chimeric Antigen Receptor Therapy for Cancer Annual Review of Medicine Vol.* 65: 333-347 (2014).

[0230] Among additional nucleic acids, e.g., genes for introduction are those to improve the efficacy of therapy, such as by promoting viability and/or function of transferred cells; genes to provide a genetic marker for selection and/or evaluation of the cells, such as to assess *in vivo* survival or localization; genes to improve safety, for example, by making the cell susceptible to negative selection in vivo as described by Lupton S. D. et al., *Mol. and Cell Biol.*, 11:6 (1991); and Riddell et al., *Human Gene Therapy* 3:319-338 (1992); see also the publications of PCT/US91/08442 and PCT/US94/05601 by Lupton et al. describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable marker with a negative selectable marker. See, e.g., Riddell et al., US Patent No. 6,040,177, at columns 14-17.

IV. Compositions, Formulations and Methods of Administration

[0231] Also provided are compositions containing the chimeric receptor, such as CAR, and compositions containing the engineered cells, including pharmaceutical compositions and formulations. Also provided are methods of using and uses of the compositions, such as in the treatment of diseases, conditions, and disorders in which the antigen is expressed, or in detection, diagnostic, and prognostic methods.

1. Compositions/Formulations

[0232] The term “pharmaceutical formulation” refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

[0233] A “pharmaceutically acceptable carrier” refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[0234] In some aspects, the choice of carrier is determined in part by the particular cell and/or by the method of administration. Accordingly, there are a variety of suitable formulations. For example, the pharmaceutical composition can contain preservatives. Suitable preservatives may include, for example, methylparaben, propylparaben, sodium benzoate, and

benzalkonium chloride. In some aspects, a mixture of two or more preservatives is used. The preservative or mixtures thereof are typically present in an amount of about 0.0001% to about 2% by weight of the total composition. Carriers are described, e.g., by Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980). Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG).

[0235] Buffering agents in some aspects are included in the compositions. Suitable buffering agents include, for example, citric acid, sodium citrate, phosphoric acid, potassium phosphate, and various other acids and salts. In some aspects, a mixture of two or more buffering agents is used. The buffering agent or mixtures thereof are typically present in an amount of about 0.001% to about 4% by weight of the total composition. Methods for preparing administrable pharmaceutical compositions are known. Exemplary methods are described in more detail in, for example, Remington: The Science and Practice of Pharmacy, Lippincott Williams & Wilkins; 21st ed. (May 1, 2005).

[0236] The formulation or composition may also contain more than one active ingredients useful for the particular indication, disease, or condition being treated with the cells, preferably those with activities complementary to the cell, where the respective activities do not adversely affect one another. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended. Thus, in some embodiments, the pharmaceutical composition further includes other pharmaceutically active agents or drugs, such as chemotherapeutic agents, e.g., asparaginase, busulfan, carboplatin, cisplatin, daunorubicin,

doxorubicin, fluorouracil, gemcitabine, hydroxyurea, methotrexate, paclitaxel, rituximab, vinblastine, vincristine, etc.

[0237] The pharmaceutical composition in some embodiments contains cells in amounts effective to treat or prevent the disease or condition, such as a therapeutically effective or prophylactically effective amount. Therapeutic or prophylactic efficacy in some embodiments is monitored by periodic assessment of treated subjects. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful and can be determined. The desired dosage can be delivered by a single bolus administration of the composition, by multiple bolus administrations of the composition, or by continuous infusion administration of the composition.

[0238] The cells may be administered using standard administration techniques, formulations, and/or devices. Provided are formulations and devices, such as syringes and vials, for storage and administration of the compositions. Administration of the cells can be autologous or heterologous. For example, immunoresponsive cells or progenitors can be obtained from one subject, and administered to the same subject or a different, compatible subject. Peripheral blood derived immunoresponsive cells or their progeny (e.g., in vivo, ex vivo or in vitro derived) can be administered via localized injection, including catheter administration, systemic injection, localized injection, intravenous injection, or parenteral administration. When administering a therapeutic composition (e.g., a pharmaceutical composition containing a genetically modified immunoresponsive cell), it will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion).

[0239] Formulations include those for oral, intravenous, intraperitoneal, subcutaneous, pulmonary, transdermal, intramuscular, intranasal, buccal, sublingual, or suppository administration. In some embodiments, the cell populations are administered parenterally. The term “parenteral,” as used herein, includes intravenous, intramuscular, subcutaneous, rectal, vaginal, and intraperitoneal administration. In some embodiments, the cell populations are administered to a subject using peripheral systemic delivery by intravenous, intraperitoneal, or subcutaneous injection.

[0240] Compositions in some embodiments are provided as sterile liquid preparations, e.g., isotonic aqueous solutions, suspensions, emulsions, dispersions, or viscous compositions, which may in some aspects be buffered to a selected pH. Liquid preparations are normally easier to

prepare than gels, other viscous compositions, and solid compositions. Additionally, liquid compositions are somewhat more convenient to administer, especially by injection. Viscous compositions, on the other hand, can be formulated within the appropriate viscosity range to provide longer contact periods with specific tissues. Liquid or viscous compositions can comprise carriers, which can be a solvent or dispersing medium containing, for example, water, saline, phosphate buffered saline, polyoi (for example, glycerol, propylene glycol, liquid polyethylene glycol) and suitable mixtures thereof.

[0241] Sterile injectable solutions can be prepared by incorporating the cells in a solvent, such as in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose, dextrose, or the like. The compositions can also be lyophilized. The compositions can contain auxiliary substances such as wetting, dispersing, or emulsifying agents (e.g., methylcellulose), pH buffering agents, gelling or viscosity enhancing additives, preservatives, flavoring agents, colors, and the like, depending upon the route of administration and the preparation desired. Standard texts may in some aspects be consulted to prepare suitable preparations.

[0242] Various additives which enhance the stability and sterility of the compositions, including antimicrobial preservatives, antioxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0243] The formulations to be used for in vivo administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

2. Methods of Administration

[0244] Provided are methods of administering the cells, populations, and compositions, and uses of such cells, populations, and compositions to treat or prevent diseases, conditions, and disorders, including cancers. In some embodiments, the cells, populations, and compositions are administered to a subject or patient having the particular disease or condition to be treated, e.g., via adoptive cell therapy, such as adoptive T cell therapy. In some embodiments, provided cells and compositions are administered to a subject, such as a subject having or at risk for the disease or condition. In some aspects, the methods thereby treat, e.g., ameliorate one or more symptom

of, the disease or condition, such as by lessening tumor burden in a cancer expressing an antigen recognized by an engineered T cell.

[0245] Methods for administration of cells for adoptive cell therapy are known and may be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; US Patent No. 4,690,915 to Rosenberg; Rosenberg (2011) *Nat Rev Clin Oncol*. 8(10):577-85). See, e.g., Themeli et al. (2013) *Nat Biotechnol*. 31(10): 928-933; Tsukahara et al. (2013) *Biochem Biophys Res Commun* 438(1): 84-9; Davila et al. (2013) *PLoS ONE* 8(4): e61338.

[0246] As used herein, a “subject” is a mammal, such as a human or other animal, and typically is human. In some embodiments, the subject, e.g., patient, to whom the cells, cell populations, or compositions are administered is a mammal, typically a primate, such as a human. In some embodiments, the primate is a monkey or an ape. The subject can be male or female and can be any suitable age, including infant, juvenile, adolescent, adult, and geriatric subjects. In some embodiments, the subject is a non-primate mammal, such as a rodent.

[0247] As used herein, “treatment” (and grammatical variations thereof such as “treat” or “treating”) refers to complete or partial amelioration or reduction of a disease or condition or disorder, or a symptom, adverse effect or outcome, or phenotype associated therewith. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis. The terms do not imply complete curing of a disease or complete elimination of any symptom or effect(s) on all symptoms or outcomes.

[0248] As used herein, “delaying development of a disease” means to defer, hinder, slow, retard, stabilize, suppress and/or postpone development of the disease (such as cancer). This delay can be of varying lengths of time, depending on the history of the disease and/or individual being treated. As is evident to one skilled in the art, a sufficient or significant delay can, in effect, encompass prevention, in that the individual does not develop the disease. For example, a late stage cancer, such as development of metastasis, may be delayed.

[0249] “Preventing,” as used herein, includes providing prophylaxis with respect to the occurrence or recurrence of a disease in a subject that may be predisposed to the disease but has not yet been diagnosed with the disease. In some embodiments, the provided cells and compositions are used to delay development of a disease or to slow the progression of a disease.

[0250] As used herein, to “suppress” a function or activity is to reduce the function or activity when compared to otherwise same conditions except for a condition or parameter of interest, or alternatively, as compared to another condition. For example, cells that suppress tumor growth reduce the rate of growth of the tumor compared to the rate of growth of the tumor in the absence of the cells.

[0251] An “effective amount” of an agent, e.g., a pharmaceutical formulation, cells, or composition, in the context of administration, refers to an amount effective, at dosages/amounts and for periods of time necessary, to achieve a desired result, such as a therapeutic or prophylactic result.

[0252] A “therapeutically effective amount” of an agent, e.g., a pharmaceutical formulation or cells, refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result, such as for treatment of a disease, condition, or disorder, and/or pharmacokinetic or pharmacodynamic effect of the treatment. The therapeutically effective amount may vary according to factors such as the disease state, age, sex, and weight of the subject, and the populations of cells administered. In some embodiments, the provided methods involve administering the cells and/or compositions at effective amounts, e.g., therapeutically effective amounts.

[0253] A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

[0254] The disease or condition that is treated can be any in which expression of an antigen is associated with and/or involved in the etiology of a disease condition or disorder, e.g. causes, exacerbates or otherwise is involved in such disease, condition, or disorder. Exemplary diseases and conditions can include diseases or conditions associated with malignancy or transformation of cells (e.g. cancer), autoimmune or inflammatory disease, or an infectious disease, e.g. caused by a bacterial, viral or other pathogen. Exemplary antigens, which include antigens associated with various diseases and conditions that can be treated, are described above.

In particular embodiments, the chimeric antigen receptor or transgenic TCR specifically binds to an antigen associated with the disease or condition.

[0255] In some embodiments, the disease or condition is a tumor, such as a solid tumor, lymphoma, leukemia, blood tumor, metastatic tumor, or other cancer or tumor type.

[0256] In some embodiments, the disease or condition is an infectious disease or condition, such as, but not limited to, viral, retroviral, bacterial, and protozoal infections, immunodeficiency, Cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenovirus, BK polyomavirus. In some embodiments, the disease or condition is an autoimmune or inflammatory disease or condition, such as arthritis, e.g., rheumatoid arthritis (RA), Type I diabetes, systemic lupus erythematosus (SLE), inflammatory bowel disease, psoriasis, scleroderma, autoimmune thyroid disease, Grave's disease, Crohn's disease, multiple sclerosis, asthma, and/or a disease or condition associated with transplant.

[0257] In some embodiments, the antigen associated with the disease or disorder is selected from the group consisting of orphan tyrosine kinase receptor ROR1, tEGFR, Her2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, and hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, OEPHa2, ErbB2, 3, or 4, FBP, fetal acetylcholine receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, Lewis Y, L1-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, ROR1, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, Her2/neu, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, and MAGE A3, CE7, Wilms Tumor 1 (WT-1), a cyclin, such as cyclin A1 (CCNA1), and/or biotinylated molecules, and/or molecules expressed by HIV, HCV, HBV or other pathogens.

[0258] Thus, the provided methods and uses include methods and uses for adoptive cell therapy. In some embodiments, the methods include administration of the cells or a composition containing the cells to a subject, tissue, or cell, such as one having, at risk for, or suspected of having the disease, condition or disorder. In some embodiments, the cells, populations, and compositions are administered to a subject having the particular disease or condition to be treated, e.g., via adoptive cell therapy, such as adoptive T cell therapy. In some embodiments, the cells or compositions are administered to the subject, such as a subject having or at risk for the disease or condition, ameliorate one or more symptom of the disease or condition.

[0259] In some embodiments, the cell therapy, e.g., adoptive T cell therapy, is carried out by autologous transfer, in which the cells are isolated and/or otherwise prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some aspects, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells, following isolation and processing are administered to the same subject.

[0260] In some embodiments, the cell therapy, e.g., adoptive T cell therapy, is carried out by allogeneic transfer, in which the cells are isolated and/or otherwise prepared from a subject other than a subject who is to receive or who ultimately receives the cell therapy, e.g., a first subject. In such embodiments, the cells then are administered to a different subject, e.g., a second subject, of the same species. In some embodiments, the first and second subjects are genetically identical. In some embodiments, the first and second subjects are genetically similar. In some embodiments, the second subject expresses the same HLA class or super type as the first subject. The cells can be administered by any suitable means. Dosing and administration may depend in part on whether the administration is brief or chronic. Various dosing schedules include but are not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion.

[0261] In certain embodiments, the cells, or individual populations of sub-types of cells, are administered to the subject at a range of about one million to about 100 billion cells and/or that amount of cells per kilogram of body weight, such as, e.g., 1 million to about 50 billion cells (e.g., about 5 million cells, about 25 million cells, about 500 million cells, about 1 billion cells, about 5 billion cells, about 20 billion cells, about 30 billion cells, about 40 billion cells, or a range defined by any two of the foregoing values), such as about 10 million to about 100 billion cells (e.g., about 20 million cells, about 30 million cells, about 40 million cells, about 60 million cells, about 70 million cells, about 80 million cells, about 90 million cells, about 10 billion cells, about 25 billion cells, about 50 billion cells, about 75 billion cells, about 90 billion cells, or a range defined by any two of the foregoing values), and in some cases about 100 million cells to about 50 billion cells (e.g., about 120 million cells, about 250 million cells, about 350 million cells, about 450 million cells, about 650 million cells, about 800 million cells, about 900 million cells, about 3 billion cells, about 30 billion cells, about 45 billion cells) or any value in between these ranges and/or per kilogram of body weight. Again, dosages may vary depending on attributes particular to the disease or disorder and/or patient and/or other treatments.

[0262] In some embodiments, the cells are administered as part of a combination treatment, such as simultaneously with or sequentially with, in any order, another therapeutic intervention, such as an antibody or engineered cell or receptor or agent, such as a cytotoxic or therapeutic agent. The cells in some embodiments are co-administered with one or more additional therapeutic agents or in connection with another therapeutic intervention, either simultaneously or sequentially in any order. In some contexts, the cells are co-administered with another therapy sufficiently close in time such that the cell populations enhance the effect of one or more additional therapeutic agents, or vice versa. In some embodiments, the cells are administered prior to the one or more additional therapeutic agents. In some embodiments, the cells are administered after the one or more additional therapeutic agents. In some embodiments, the one or more additional agents includes a cytokine, such as IL-2, for example, to enhance persistence. In some embodiments, the methods comprise administration of a chemotherapeutic agent.

[0263] Following administration of the cells, the biological activity of the engineered cell populations in some embodiments is measured, e.g., by any of a number of known methods. Parameters to assess include specific binding of an engineered or natural T cell or other immune cell to antigen, *in vivo*, e.g., by imaging, or *ex vivo*, e.g., by ELISA or flow cytometry. In certain embodiments, the ability of the engineered cells to destroy target cells can be measured using any suitable method known in the art, such as cytotoxicity assays described in, for example, Kochenderfer et al., J. Immunotherapy, 32(7): 689-702 (2009), and Herman et al. J. Immunological Methods, 285(1): 25-40 (2004). In certain embodiments, the biological activity of the cells is measured by assaying expression and/or secretion of one or more cytokines, such as CD 107a, IFN γ , IL-2, and TNF. In some aspects the biological activity is measured by assessing clinical outcome, such as reduction in tumor burden or load.

[0264] In certain embodiments, the engineered cells are further modified in any number of ways, such that their therapeutic or prophylactic efficacy is increased. For example, the engineered CAR or TCR expressed by the population can be conjugated either directly or indirectly through a linker to a targeting moiety. The practice of conjugating compounds, e.g., the CAR or TCR, to targeting moieties is known in the art. See, for instance, Wadwa et al., J. Drug Targeting 3: 111 (1995), and U.S. Patent 5,087,616.

V. EXEMPLARY EMBODIMENTS

[0265] Among the provided embodiments are:

1. A variant chimeric receptor, comprising a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein:

the reference chimeric receptor comprises a first domain and a second domain, joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor comprises up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and

a peptide fragment having the sequence of an 8-15 amino acid portion of the modified junction region has a binding affinity for a human leukocyte antigen (HLA) molecule that is lower than the binding affinity, for the same HLA molecule, of a peptide fragment having the sequence of the corresponding portion of the junction region of the reference chimeric receptor.

2. The variant chimeric receptor of embodiment 1, wherein the peptide fragment of the corresponding portion of the junction region of the reference chimeric receptor has a binding affinity of less than 1000 nM, less than 500 nM or less than 50 nM.

3. A variant chimeric receptor, comprising a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein:

the reference chimeric receptor comprises a first domain and a second domain, joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor comprises up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and

the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the modified junction region for a human HLA molecule is lower than the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the junction region of the reference chimeric receptor.

4. The variant chimeric receptor of any of embodiments 1-3, wherein the binding affinity or average of binding affinities is more than 2-fold, more than 5-fold, more than 10-fold, more than 25-fold, more than 50-fold or more than 100-fold lower.

5. A variant chimeric receptor, comprising a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein:

the reference chimeric receptor comprises a first domain directly linked to a second domain joined in contiguous sequence at a junction, wherein;

the junction region of the reference chimeric receptor comprises up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and

the number of peptide fragments having the sequence of an 8-15 amino acid portion of the modified junction region that has a binding affinity for a human leukocyte antigen (HLA) of less than 1000 nM is reduced compared to the number of peptide fragments having the sequence of an 8-15 amino acid portion of the junction region of the reference chimeric receptor that has the same affinity for binding the same HLA.

6. The variant chimeric receptor of embodiment 5, wherein:

the number of peptide fragments within the modified junction region that exhibits a binding affinity for an HLA of less than 500 nM or less than 50 nM is reduced; or

the binding affinity of less than 1000 nM is a binding affinity of less than 500 nM or less than 50 nm.

7. The variant chimeric receptor of any of embodiments 1-6, wherein the binding affinity is an IC₅₀ and the comparison of binding of peptide fragments of the modified junction regions and the peptide fragments of the junction region of the reference chimeric receptor is with reference to the same standard peptide.

8. The variant chimeric receptor of any of embodiments 1-7, wherein:

the first domain and/or second domain comprises a domain of a natural human protein; and/or

the first domain and/or second domain comprises an extracellular binding domain, a hinge domain, a transmembrane domain, or an intracellular signaling domain, which intracellular signaling domain is, optionally, a costimulatory signaling domain or an activating cytoplasmic signaling domain.

9. The variant chimeric receptor of any of embodiments 1-8, wherein the first domain and second domain are not present in the same molecule *in vivo* in a human subject.

10. The variant chimeric receptor of any of embodiments 1-9, wherein the first domain and second domain are, respectively, an extracellular ligand binding domain and a hinge domain, a hinge domain and a transmembrane domain, a transmembrane domain and an intracellular costimulatory signaling domain, and an intracellular costimulatory signaling domain and an activating cytoplasmic signaling domain, which can include functional portions of such domains.

11. The variant chimeric receptor of any of embodiments 1-10, wherein the first domain is a transmembrane domain or a functional portion thereof and the second domain is a costimulatory signaling domain or a functional portion thereof.

12. The variant chimeric receptor of embodiment 11, wherein the transmembrane domain is a CD28 transmembrane domain or a functional portion or variant thereof and the costimulatory signaling domain is a 4-1BB signaling domain or a functional portion or variant thereof.

13. The variant chimeric receptor of any of embodiments 1-12, wherein the junction region of the reference chimeric receptor comprises up to 13 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction.

14. The variant chimeric receptor of any of embodiments 1-13, wherein the peptide fragment(s) comprises a sequence of amino acids between or between about 8 and 15 amino acids in length, or comprises a sequence of amino acids that is at least or at least about or is or is about 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in length.

15. The variant chimeric receptor of any of embodiments 1-14, comprising:
a domain of at least 95% sequence identity to the first domain and/or a domain of at least 95% sequence identity to the second domain;
a domain identical in sequence to the first domain and a domain of at least 95% sequence identity to the second domain; or
a domain of at least 95% sequence identity to the first domain and a domain identical in sequence to the second domain,

wherein at least one or both of the domains present in the variant chimeric receptor is modified compared to the first domain and/or second domain of the reference chimeric receptor in the portion comprising the modified junction region.

16. The variant chimeric receptor of any of embodiments 1-15, wherein:

the variant chimeric receptor comprises at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the reference chimeric receptor; and/or

the variant chimeric receptor comprises up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid modifications compared to the reference chimeric receptor.

17. The variant chimeric receptor of any of embodiments 12-16, wherein the CD28 transmembrane domain comprises the sequence of amino acids set forth in SEQ ID NO:2, 103 or 104 or a functional portion or variant thereof comprising a sequence that exhibits at least 95% sequence identity to SEQ ID NO:2, 103 or 104; and

the 4-1BB costimulatory signaling domain comprises the sequence of amino acids set forth in SEQ ID NO:3 or a functional portion or variant thereof comprising a sequence that exhibits at least 95% sequence identity to SEQ ID NO:3.

18. The variant chimeric receptor of any of embodiments 12-17, wherein the first domain and second domain together comprise the sequence of amino acids set forth in SEQ ID NO:5 or a functional portion or variant thereof comprising a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:5.

19. The variant chimeric receptor of any of embodiments 12-18, wherein the first domain and second domain together comprise the sequence of amino acids set forth in SEQ ID NO:5.

20. The variant chimeric receptor of any of embodiments 12-19, wherein the one or more modifications are within a portion between residue 13 and 42 or between amino acid residue 15 and 40, with reference to numbering set forth in SEQ ID NO:5.

21. The variant chimeric receptor of any of embodiments 1-20, wherein the one or more modifications comprises an amino acid insertion, replacement or deletion and/or wherein each of the one or more modifications individually comprises an amino acid insertion, replacement, or deletion.

22. The variant chimeric receptor of any of embodiments 1-21, wherein the first and/or second domain comprises a transmembrane domain, wherein

the one or more modifications is not or does not comprise a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, which optionally is the CD28 transmembrane domain; or

the one or more modifications comprises a modification of a hydrophobic amino acid residue within the transmembrane domain, which optionally is the CD28 transmembrane domain, wherein the modification is or comprises a substitution of the hydrophobic amino acid with another different hydrophobic amino acid residue; or

the one or more modifications is not or does not comprise a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, which optionally is the CD28 transmembrane domain, other than a substitution with another hydrophobic amino acid residue.

23. The variant chimeric receptor of any of embodiments 12-22, wherein the one or more modifications comprise a modification at an amino acid residue corresponding to an amino acid residue between residues 28 and 42 with reference to numbering set forth in SEQ ID NO:5.

24. The variant chimeric receptor of any of embodiments 1-23, wherein the amino acid modification is an insertion and the variant chimeric receptor comprises an insertion between amino acid residues adjacent to the junction between the domains, which optionally corresponds to amino acid residues 27 and 28 with reference to numbering set forth SEQ ID NO:5.

25. The variant chimeric receptor of embodiment 24, comprising insertion of 1, 2, 3, 4 or 5 amino acid residues.

26. The variant chimeric receptor of embodiment 24 or embodiment 25, wherein the insertion is of any amino acid residue, which, optionally, is asparagine (N).

27. The variant chimeric receptor of any of embodiments 12-26, wherein the one or more modifications comprises an amino acid replacement(s) and the amino acid replacement(s) are at one or more residues corresponding to a residue selected from 28, 31 or 34 with reference to numbering set forth in SEQ ID NO:5.

28. The variant chimeric receptor of embodiment 27, wherein the amino acid replacement(s) is to any other amino acid residue, which, optionally, is selected from among leucine (L), asparagine (N), glutamine (Q), alanine (A), serine (S) or histidine (H).

29. The variant chimeric receptor of embodiment 27 or embodiment 28, wherein the amino acid replacement(s) corresponds to or is a replacement selected from among K28A, K28H, K28L, K28Q, K28S, R31A, R31H, R31L, R31N, R31S, L34A and L34S, with reference to numbering set forth in SEQ ID NO:5.

30. The variant chimeric receptor of any of embodiments 27-29, wherein the amino acid replacement does not comprise the single amino acid replacement corresponding to L34A or L34S, with reference to numbering set forth in SEQ ID NO:5.

31. The variant chimeric receptor of any of embodiments 27-30, wherein the amino acid replacements are or correspond to amino acid replacements selected from among K28Q/R31A, K28Q/R31N, K28Q/R31S, K28Q/L34A, K28Q/L34S, R31N/L34A, R31N/L34S, K28Q/R31N/L34A, K28Q/R31N/L34S.

32. The variant chimeric receptor of any of embodiments 1-31, wherein:
the variant chimeric receptor comprises a modified junction region comprising less than 100% sequence identity to SEQ ID NO:137 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% to SEQ ID NO:137 and comprises the modifications; and/or
the variant chimeric receptor comprises a sequence comprising less than 100% sequence identity to SEQ ID NO:5 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% to SEQ ID NO:5 and comprises the modifications.

33. The variant chimeric receptor of any of embodiments 1-32, wherein the variant chimeric receptor comprises a modified junction region selected from among:

- i) the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184;
- ii) a functional variant thereof comprising a sequence of amino acids that exhibits at least 95% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184 and that comprise the modification(s); or
- iii) a functional portion of i) or ii) and comprises the modification(s).

34. The variant chimeric receptor of any of embodiments 1-33, wherein the variant chimeric receptor comprises:

- i) the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183;
- ii) a functional variant thereof comprising a sequence of amino acids that exhibits at least 95% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183 and that comprise the modification(s);
- iii) or is a functional portion of i) or ii) and comprises the modification(s).

35. The variant chimeric receptor of any of embodiments 1-34, wherein the modified junction region comprises no more than 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications compared to the junction region of the reference chimeric receptor.

36. The variant chimeric receptor of any of embodiments 1-35, wherein the first domain or second domain is a transmembrane domain and the corresponding domain in the variant chimeric receptor comprises a substantially hydrophobic hydropathy profile and/or comprises a grand average of hydropathy (GRAVY) value of greater than 0, 0.25, 0.5, 0.75, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 or greater.

37. The variant chimeric receptor of any of embodiments 1-36, wherein first domain or second domain comprises an intracellular signaling domain and the corresponding domain in the variant chimeric receptor is capable of inducing the activation or cellular localization of a TRAF and/or is capable of inducing TRAF-mediated signaling.

38. The variant chimeric receptor of embodiment 37, wherein the intracellular signaling domain is a 4-1BB co-stimulatory signaling domain and/or the TRAF is selected from among TRAF1, TRAF2 or TRAF3.

39. The variant chimeric receptor of any of embodiments 12-38, wherein the variant chimeric receptor comprises amino acids TTQE at positions corresponding to 49-52 and/or amino acids PEEE at positions corresponding to residues 60-63, each with reference to numbering set forth in SEQ ID NO:5.

40. The variant chimeric receptor of any of embodiments 1-39, wherein the HLA is an HLA class I and/or an HLA class II.

41. The variant chimeric receptor of any of embodiments 1-40, wherein the HLA class I is selected from an HLA allele set forth in Table 1A and/or the HLA class II allele is selected from an HLA allele set forth in Table 1B.

42. The variant chimeric receptor of any of embodiments 1-41, wherein the HLA class I allele is selected from among HLA-A*02:01, HLA-A*03:01, HLA-A*11:01 and HLA-B*08:01.

43. The variant chimeric receptor of any of embodiments 3-42, wherein the HLA comprises a plurality of HLA molecules and average binding affinities to one or more of the plurality of HLA molecules is lower and/or the number of peptide fragments that individually bind to one or more of the plurality of HLA molecules is reduced.

44. The variant chimeric receptor of embodiment 43, wherein the plurality of HLA molecules is selected from:

a plurality of HLA class I molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class I molecules in the worldwide population or in the Caucasian population;

a plurality of HLA class II molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class II molecules in the worldwide population or in the Caucasian population; or

a plurality of HLA class I molecules and HLA class II molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class I molecules and HLA class II molecules in the worldwide population or in the Caucasian population;

45. The variant chimeric receptor of any of embodiments 1-44, wherein the binding affinity is as determined *in vitro*.

46. The variant chimeric receptor of any of embodiments 1-45 that exhibits reduced immunogenicity compared to the reference chimeric receptor upon administration to a human subject, optionally wherein the subject has received administration of the reference chimeric receptor.

47. The variant chimeric receptor of embodiment 46, wherein the reduced immunogenicity comprises a reduced CD4+ T cell immune response and/or a reduced CD8+ T cell immune response.

48. The variant chimeric receptor of any of embodiments 11-47, wherein:
the reference chimeric receptor further comprises an extracellular ligand-binding domain; and/or

the variant chimeric receptor further comprises an extracellular ligand-binding domain.

49. The variant chimeric receptor of embodiment 48 that is a chimeric antigen receptor (CAR), wherein the ligand-binding domain is an antigen-binding domain.

50. The variant chimeric receptor of embodiment 49, wherein the antigen-binding domain is an antibody or an antibody fragment.

51. The variant chimeric receptor of embodiment 50, wherein the antigen-binding domain is an antibody fragment that is a single chain fragment.

52. The variant chimeric receptor of embodiment 50 or embodiment 51, wherein the fragment comprises antibody variable regions joined by a flexible immunoglobulin linker.

53. The variant chimeric receptor of any of embodiments 50-52, wherein the antibody fragment comprises an scFv.

54. The variant chimeric receptor of any of embodiments 48-52, wherein the ligand-binding domain specifically binds an antigen that is associated with a disease or disorder.

55. The variant chimeric receptor of embodiment 54, wherein:

the disease or disorder is an infectious disease or condition, an autoimmune disease, an inflammatory disease or a tumor or a cancer;

the ligand-binding domain specifically binds to a tumor antigen; and/or

the ligand-binding domain specifically binds to an antigen selected from the group consisting of ROR1, Her2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, EPHA2, ErbB2, ErbB3, ErbB4, FBP, fetal acetylcholine receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, Lewis Y, L1-cell adhesion molecule, MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, MAGE A3, CE7, Wilms Tumor 1 (WT-1) and cyclin A1 (CCNA1).

56. The variant chimeric receptor of any of embodiments 11-55, wherein:

the reference chimeric receptor further comprises an activating cytoplasmic signaling domain; and/or

the variant chimeric receptor further comprises an activating cytoplasmic domain.

57. The variant chimeric receptor of embodiment 56, wherein the activating cytoplasmic domain comprises a T cell receptor (TCR) component and/or comprise an immunoreceptor tyrosine-based activation motif (ITAM).

58. The variant chimeric receptor of embodiment 56 or embodiment 57, wherein the activating cytoplasmic signaling domain is or comprises a cytoplasmic signaling domain of a zeta chain of a CD3-zeta (CD3 ζ) chain or a functional variant or signaling portion thereof.

59. The variant chimeric receptor of any of embodiments 11-58, wherein:

the reference chimeric receptor comprises from its N to C terminus in order: an extracellular ligand-binding domain, the first domain that is a transmembrane domain, the second domain that is an intracellular costimulatory domain and an activating cytoplasmic signaling domain; and/or

the variant chimeric receptor comprises from its N to C terminus in order: an extracellular ligand-binding domain, a transmembrane domain, a intracellular costimulatory domain and an activating cytoplasmic signaling domain, wherein the transmembrane domain and intracellular costimulatory domain are joined in contiguous sequence at a junction to form the modified junction region.

60. A nucleic acid molecule encoding the variant chimeric receptor of any of embodiments 1-59.
61. A vector, comprising the nucleic acid molecule of embodiment 60.
62. The vector of embodiment 61 that is a viral vector.
63. The vector of embodiment 61 or embodiment 62 that is a retroviral vector, which optionally is a lentiviral vector or a gammaretroviral vector.
64. An engineered cell, comprising the nucleic acid of embodiment 60 or the vector of any of embodiments 61-63 or expressing the chimeric receptor of any of embodiments 1-59.
65. The engineered cell of embodiment 64, which is a T cell.
66. The engineered cell of embodiment 64 or embodiment 65 that is a CD4⁺ T cell and/or a CD8⁺ T cell.
67. A composition, comprising the engineered cells of any of embodiments 64-66, and optionally a pharmaceutically acceptable buffer.
68. A method of treatment, comprising administering the cell of any of embodiments 64-66 or the composition of embodiment 67 to a subject having a disease or condition.
69. The method of embodiment 68, wherein the chimeric receptor specifically binds to a ligand or antigen associated with the disease or condition.
70. The method of embodiment 68 or embodiment 69, wherein the disease or condition is a cancer, a tumor, an autoimmune disease or disorder, or an infectious disease.
71. The method of any of embodiments 68-70, wherein the genetically engineered T cells in the composition exhibit increased or longer expansion and/or persistence in the subject than in a subject administered the same or about the same dosage amount of a reference cell composition expressing the reference chimeric receptor.
72. The method of embodiment 71, wherein the increase is at least 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, or 5-fold.

73. The method of embodiment 71 or embodiment 72, wherein the increase is observed or is present within a month, within two months, within six months or within one year of administering the cells.

VI. DEFINITIONS

[0266] The terms “polypeptide” and “protein” are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Polypeptides, including the provided receptors and other polypeptides, e.g., linkers or peptides, may include amino acid residues including natural and/or non-natural amino acid residues. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, and phosphorylation. In some aspects, the polypeptides may contain modifications with respect to a native or natural sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0267] As used herein, “percent (%) sequence identity” and “percent identity” when used with respect to a nucleotide sequence (reference nucleotide sequence) is defined as the percentage of nucleotide residues in a candidate sequence (e.g., the subject chimeric receptor, such as a variant chimeric receptor) that are identical with the nucleotide residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0268] As used herein, “corresponding” with reference to a position of a region, such as “at a position corresponding to” or recitation that a region or amino acid positions “correspond to” a region or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm, such as the GAP algorithm. Exemplary described corresponding residues can be identified by alignment of a sequence with an exemplary reference junction region sequence set forth in SEQ ID NO:5. By aligning the

sequences, one skilled in the art can identify corresponding residues or regions, for example, using conserved and identical amino acid residues as guides. In general, to identify corresponding positions or regions, the sequences of amino acids are aligned so that the highest order match is obtained (see, e.g. : Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; Carrillo et al. (1988) SIAM J Applied Math 48: 1073).

[0269] The term “vector,” as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as “expression vectors.” Among the vectors are viral vectors, such as lentiviral vectors.

[0270] The terms “host cell,” “host cell line,” and “host cell culture” are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include “transformants” and “transformed cells,” which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell are included herein.

[0271] As used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. For example, “a” or “an” means “at least one” or “one or more.” It is understood that aspects and variations described herein include “consisting” and/or “consisting essentially of” aspects and variations.

[0272] Throughout this disclosure, various aspects of the claimed subject matter are presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the claimed subject matter. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual

numerical values within that range. For example, where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the claimed subject matter. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the claimed subject matter, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the claimed subject matter. This applies regardless of the breadth of the range.

[0273] The term “about” as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to “about X” includes description of “X”.

[0274] As used herein, a composition refers to any mixture of two or more products, substances, or compounds, including cells. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

VII. Examples

[0275] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1: Analysis of Transgene Product-Specific Host Immune Responses

[0276] Pre- and post-treatment peripheral blood mononuclear cells (PBMC) samples were obtained from four (4) subjects with B cell malignancies treated with autologous T cells expressing a CD19-specific CAR. The CAR included an anti-CD19 scFv derived from murine antibody, a hinge domain, a CD28 transmembrane domain, a 4-1BB intracellular signaling domain, and a CD3-zeta intracellular signaling domain. The CAR-expressing T cells also expressed a truncated EGFR (EGFRt) as a surrogate marker by transducing cells with a lentiviral vector containing a nucleic acid encoding the CAR and a nucleic acid encoding the EGFRt surrogate marker, separated by a T2A ribosome switch domain.

[0277] Pre- and post (day 42)-infusion PBMCs obtained from the subjects were assessed to detect the presence or absence of specific anti-CAR immune responses essentially as described by Berger et al. *Blood*. 2006 March; 107(6): 2294-2302, Berger et al. *J Virol*. 2001 January

75(2): 799-808, Riddell et al. *Nature Medicine*. 1996 February 2(2): 216-223, Berger et al. *Blood*. 2005 February 105(4): 1640-1647. Briefly, PBMCs (responders) were stimulated *in vitro* with autologous gamma-irradiated cells transduced with the CAR expressed by the administered cells (stimulators at a 1:1 or 2:1 responder-to-stimulator ratio). The cultures then were assessed in a chromium release assay for cytotoxicity against autologous ⁵¹Cr-labeled CAR-transduced ("CD19 CAR") and non-transduced ("Mock") T cells (targets) at various effector-to-target (E/T) ratios. Following co-incubation, release of chromium was quantified and the percentage of maximum achievable lysis in each sample determined.

[0278] The results for samples derived from one exemplary patient are shown in Figure 1, which depicts the cytolytic activity of PBMCs pre-infusion and post-infusion at day 42. Whereas no cytolytic activity specific for CAR-transduced target cells was detected in any pre-infusion PBMC-derived cultures, in two of the four subjects assessed, CAR-specific lytic activity was detected in cultures derived from post-infusion PBMC samples. These results indicate that CAR-specific immune responses can develop following a single infusion of CAR-expressing T cells.

[0279] Epitope mapping was carried out to assess region(s) of the CAR recognized by the specific immune responses. Pre- and post-infusion PBMC samples were stimulated in the presence of individual pools of multiple 15-mer peptides, with sequences representing overlapping portions (11 amino acid overlap) of the entire length of an approximately 500 amino acid sequence of the CAR expressed by the administered cells. Cells were stained with antibodies to detect CD8 and CD4 surface expression and intracellular expression of cytokine. Twenty-three (23) pools were assessed, each containing ten (10) peptides each and collectively including 125 individual overlapping peptides, with each peptide represented in at least two of the pools.

[0280] This design permitted the generation of an analytic grid to assess responses specific for individual peptides, whereby a peptide present in more than one pool detected as hits in this assay was deemed a potentially immunogenic peptide hit. For the two patients in whom a CAR-specific immune response had been detected, six and three peptide hits, respectively, were identified.

[0281] Individual ELISpot assays were performed using an anti-cytokine capture antibody to assess the presence or absence of a specific immune response for each of these individual hits (see Berger et al. (2006); Berger et al. (2001); Riddell et al. (1996); and Berger et al. (2005),

supra). The results of an exemplary assay for one patient are shown in FIG. 2. Specific immune responses against peptides with sequences within the V_H portion of the scFv of the CAR were detected in both patients assessed (including regions within the FR1, CDR1, and FR2 regions for one patient and within the FR3 for the other). For the first patient, specific immune responses also were detected against two overlapping 15-mer peptides, each containing the junction between the transmembrane domain and costimulatory domain of the CAR (labeled “fusion site” in FIG. 2). These two overlapping 15-mer peptides had the amino acid sequences AFIIFWVKRGRKKLL (SEQ ID NO: 8) and FWVKRGRKKLLYIFK (SEQ ID NO: 9), respectively. In another study following administration with a different CAR having a murine scFv, CD28 transmembrane and costimulatory domains and a CD3 zeta domain, using similar methods, an immune response also was detected for one subject against a pool containing V_H portions of an anti-CD19 scFv and for another subject in a pool containing junction portions.

[0282] No specific immune responses were detected in the patients by this assay against peptides within other regions. For example, in this assay, no specific responses were detected against peptides having sequences within other CDRs or framework regions of the scFv, peptides within regions of costimulatory or transmembrane domain but not spanning the junction between the two, or peptides within the EGFRt or CD3- ζ region of the CAR. Specific immune responses were not detected against endogenous sequences.

Example 2: *In Silico* Analysis of Peptides Derived from Junction Regions of a CAR for Binding to HLA Class I and HLA Class II

[0283] T cell epitope prediction tools, available from the Immune Epitope Database and analysis resource (IEDB), were used for *in silico* analysis to predict MHC-binding affinities and other properties related to potential immunogenicity for each of a series of overlapping peptide sequences within a portion of an exemplary CAR sequence. The portion included a spacer having an immunoglobulin-derived hinge domain, a human CD28 transmembrane domain, a human 4-1BB costimulatory domain, and a human CD3zeta signaling domain. In the portion assessed, the hinge domain was a human IgG4 hinge domain, the CD28 transmembrane domain comprised a sequence set forth in SEQ ID NO:2 and the 4-1BB costimulatory domain contained the sequence set forth in SEQ ID NO:3. This portion thus contained three junctions between different domains derived from human sequences (which junctions may have represented sites of potential immunogenicity against a CAR upon administration to a human subject): the junction between the spacer region and transmembrane domain, the junction between the transmembrane

domain and costimulatory domain, and the junction between the costimulatory domain and intracellular signaling domain (see Figures 3A and 3B).

[0284] To identify portions of the sequence that may have particular properties making them more likely to be presented to T cells, affinities for binding to 27 individual HLA class I alleles and 56 individual HLA class II alleles were predicted for overlapping peptides along the length of the portion, of 8-14 amino acids in length and of 15 amino acids in length (containing 9-mer binding core), respectively. These alleles, collectively representing HLA alleles present in greater than 99% of the worldwide population, and their approximate frequency in the United States population are listed in Tables 1A and 1B.

Table 1A: HLA class I		
Class I	allele	Frequency in population
1	HLA-A*01:01	12.94
2	HLA-A*02:01	42.88
3	HLA-A*02:03	0.19
4	HLA-A*02:06	1.55
5	HLA-A*03:01	13.50
6	HLA-A*11:01	11.60
7	HLA-A*23:01	8.30
8	HLA-A*24:02	22.56
9	HLA-A*26:01	5.36
10	HLA-A*30:01	6.29
11	HLA-A*30:02	5.21
12	HLA-A*31:01	6.87
13	HLA-A*32:01	3.71
14	HLA-A*33:01	2.62
15	HLA-A*68:01	6.36
16	HLA-A*68:02	4.79
17	HLA-B*07:02	12.96
18	HLA-B*08:01	9.23
19	HLA-B*15:01	6.54
20	HLA-B*35:01	13.03
21	HLA-B*40:01	9.79
22	HLA-B*44:02	7.22
23	HLA-B*44:03	8.96
24	HLA-B*51:01	8.51
25	HLA-B*53:01	7.26
26	HLA-B*57:01	3.49
27	HLA-B*58:01	4.82

Table 1B: HLA class II					
Class II	allele	Frequency in population	Class II	allele	frequency in population
1	HLA-DRB1*01:01	13.62	29	HLA-DQA1*01:02/DQB1*05:02	21.13
2	HLA-DRB1*15:01	22.86	30	HLA-DQA1*01:02/DQB1*06:02	30.74
3	HLA-DRB1*03:01	21.82	31	HLA-DQA1*03:01/DQB1*03:02	31.56
4	HLA-DRB1*04:01	15.54	32	HLA-DQA1*01:02/DQB1*06:04	19.00
5	HLA-DRB1*11:01	10.92	33	HLA-DQA1*05:01/DQB1*03:01	80.58
6	HLA-DRB1*13:01	9.86	34	HLA-DQA1*02:01/DQB1*02:02	27.99
7	HLA-DRB1*07:01	19.84	35	HLA-DQA1*03:01/DQB1*03:01	49.92
8	HLA-DRB1*01:01	4.06	36	HLA-DQA1*02:01/DQB1*03:03	23.32
9	HLA-DRB1*01:02	1.85	37	HLA-DQA1*03:03/DQB1*03:03	20.22
10	HLA-DRB1*04:02	6.28	38	HLA-DPA1*01:03/DPB1*01:01	99.83
11	HLA-DRB1*04:05	1.22	39	HLA-DPA1*01:03/DPB1*02:01	99.83
12	HLA-DRB1*04:07	2.78	40	HLA-DPA1*01:03/DPB1*03:01	99.82
13	HLA-DRB1*04:08	1.26	41	HLA-DPA1*01:03/DPB1*04:01	99.88
14	HLA-DRB1*08:04	0.86	42	HLA-DPA1*01:03/DPB1*04:02	99.86
15	HLA-DRB1*09:01	5.33	43	HLA-DPA1*01:03/DPB1*05:01	99.81
16	HLA-DRB1*10:01	2.78	44	HLA-DPA1*02:01/DPB1*01:01	23.54
17	HLA-DRB1*11:02	0.94	45	HLA-DPA1*02:01/DPB1*02:01	24.11
18	HLA-DRB1*11:03	0.74	46	HLA-DPA1*02:01/DPB1*03:01	17.63
19	HLA-DRB1*11:04	4.76	47	HLA-DPA1*02:01/DPB1*04:01	46.73
20	HLA-DRB1*15:02	0.78	48	HLA-DPA1*02:01/DPB1*04:02	38.04
21	HLA-DRB1*15:03	1.22	49	HLA-DPA1*02:01/DPB1*05:01	13.24
22	HLA-DRB1*16:01	4.06	50	HLA-DPA1*02:01/DPB1*06:01	8.59
23	HLA-DRB1*16:02	0.84	51	HLA-DPA1*02:01/DPB1*09:01	7.26
24	HLA-DRB3*02:02	0.00	52	HLA-DPA1*02:01/DPB1*11:01	9.98
25	HLA-DRB3*03:01	0.00	53	HLA-DPA1*02:01/DPB1*13:01	11.55
26	HLA-DRB5*01:01	0.00	54	HLA-DPA1*02:01/DPB1*14:01	7.98
27	HLA-DQA1*01:01/DQB1*05:01	30.57	55	HLA-DPA1*02:01/DPB1*15:01	7.73
28	HLA-DQA1*05:01/DQB1*02:01	76.17	56	HLA-DPA1*02:01/DPB1*17:01	10.40

[0285] Algorithm-based T cell epitope prediction tools available from the IEDB were used to predict IC50 values for binding to HLA class I molecules for each 8-14 amino acid peptide in the dataset using ANN (Nielsen et al. (2003) Protein Sci., 12:1007-1017 and Lundegaard et al. (2008) NAR, 36:W509-512) and, in some cases, one or more additional prediction using SMM (Peters and Sette (2005) BMC Bioinformatics, 6:132) and comblib (Sidney et al. (2008) Immunome Res. 4:2, or the Consensus tool (see Kim, et al. (2012) Immune epitope database

analysis resource, NAR (combining predictions from any of the foregoing). Predictions for IC50 values for binding to HLA class II for each 15 amino acid peptide in the dataset was made using the NetMHCIIpan method (Karosiene et al. (2013) Immunogenetics 65(10):711; Nielsen et al. (2008) PLoS Comput Biol.4(7)e1000107). For each individual position within the portion of the CAR amino acid sequence, the total number of sequences in the dataset that included the position and was predicted to bind to any of the class I or class II alleles with a predicted IC50 of less than 50 nm was determined. FIGs 4A (HLA class I) and 4B (HLA class II), depict the results for class I and class II alleles, respectively, showing positional coverage along the length of the sequence, based on the determined total number, weighted according to the frequency of the individual HLA alleles in the population. The area under the curve (AUC) across the entire assessed region was approximately 1321 for HLA class I binding and 2943 for HLA class II binding. The AUC for the transmembrane-costimulatory domain region was approximately 931 for HLA class I binding and 2212 for HLA class II binding.

[0286] As shown in FIGs. 4A and 4B, certain portions of the sequence were predicted by this method to contain fragments more likely to bind well in MHC complexes and thus be presented as epitopes for potential recognition by T cells. Binding affinity for HLA alleles alone does not necessarily predict immunogenicity. Given that the individual domains (e.g., transmembrane, costimulatory) in this exemplary CAR were human-derived, upon administration to a human subject, immunogenic responses were less likely to develop against an epitope within any one of these individual regions alone (as opposed to an epitope spanning multiple regions not ordinarily associated with one another, and/or including a junction between such regions). For example, even for a peptide predicted to bind well to and be presented in the context of an MHC molecule, if the peptide was derived entirely from an endogenous protein, it may be recognized as “self” and thus may fail to induce a productive immune response. For example, whereas certain regions entirely within a single transmembrane or cytoplasmic domain scored highly on the HLA-binding affinity prediction, in the results described in Example 1, no immune responses were detected against peptide sequences solely within either one of these domains of a similar CAR sequence. Accordingly, while various “hot spots” were observed with respect to predicted HLA-binding affinity, subsequent assessment and alteration focused on those areas that not only had higher predicted IC50 values, but also included potential epitopes that spanned the junction between different domains derived from two different proteins.

[0287] In particular, a junction region that includes one or more potential peptide epitopes spanning the junction of the CD28 transmembrane domain and 4-1BB signaling domain of the exemplary CAR was further assessed. With respect to the sequence set forth in SEQ ID NO:5, which includes the exemplary human CD28 transmembrane domain (SEQ ID NO:2) and exemplary human 4-1BB costimulatory domain (SEQ ID NO:3), the assessed junction region contained 13 amino acids on either side of the junction spanning the CD28 transmembrane and 4-1BB costimulatory domains as follows:

FWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:5), in which a 26 amino acid junction region is indicated by bold, and the two amino acids just C' and N' of the junction between the domains is indicated by underline. The assessed 26 amino acid junction region is set forth in SEQ ID NO:137 and corresponds to amino acid residues 15 to 40 of the sequence of amino acids set forth in SEQ ID NO:5.

[0288] *In silico* modeling was carried out to identify one or more amino acid modifications (mutations) within the 26-amino acid junction region set forth in SEQ ID NO: 137 resulting in peptide fragments that were predicted to bind with high IC50 values to class I and class II alleles, and thus that were likely to reduce the potential for inducing immunogenicity against a CAR containing this region. Specifically, predictions were made for variant peptide fragments of the junction region containing one or more mutations at amino acid residue positions corresponding to positions 14, 17 and 20 with numbering with reference to SEQ ID NO:137 (which correspond to one or more mutations at amino acid positions corresponding to positions 28, 31 and 34 with numbering with reference to SEQ ID NO:5). In this exemplary study, these residues were chosen for further analysis following *in silico* mutagenesis and binding predictions of all high affinity epitopes in which all possible single amino acid replacements across that epitope were surveyed for their impact on the predicted IC50 values. Residues that resulted in greater IC50 predictions (decrease in the binding) were identified, which identified the above residues as being sensitive to replacements.

[0289] A series of different variant junction regions were assessed, each containing one or more amino acid replacement at the assessed position(s), as compared to the non-mutated junction region within the exemplary CAR sequence. An exemplary subset of amino acid replacements at the identified positions were chosen that may be less disruptive to the structure or function of either the transmembrane region of the costimulatory signaling domain. Also,

replacements were chosen that may be able to impact more than one epitope at a time, since the epitopes overlap. Specifically, individual variant junction regions contained the following modifications (amino acid replacements): K28A, K28H, K28L, K28Q, K28S, R31A, R31H, R31L, R31N, R31S, L34A, L34S, K28Q/R31A, K28Q/R31N, K28Q/R31S, K28Q/L34A, K28Q/L34S, R31N/L34A, R31N/L34S, K28Q/R31N/L34A, K28Q/R31N/L34S, with numbering with reference to SEQ ID NO:5.

[0290] For the non-variant and variant junction regions, weighted immunogenicity scores were obtained for class I and class II alleles, using the T cell epitope prediction tools available from IEDB. Scores were derived using predicted IC50 values for each of a series of 8-mer to 14-mer overlapping peptides (for each of the 27 HLA class I alleles, individually) and a series of 15-mer overlapping peptides (for each of the 56 HLA class II alleles, individually) within the respective (variant or non-variant) 26-amino acid junction region, and were weighted based on relative frequency in the population of the individual HLA class I and class II alleles. A higher relative score is indicative of a higher degree of predicted binding.

[0291] The results are set forth in FIG. 5. The results demonstrate the ability to decrease in the overall predicted HLA class I immunogenicity score within a CAR junction region by modifying amino acids within the region. The results also confirm the ability to reduce predicted HLA class I binding affinity (and hence reduced predicted immunogenicity score) without resulting in a substantial increase in the predicted immunogenicity score for HLA class II binding. Thus, in general, the results showed that amino acid modification(s) within a region spanning a junction between a CD28 transmembrane domain and a 4-1BB costimulatory domain of a CAR could be made and effect an overall reduction in the predicted affinity for human HLA binding, which would be consistent with a reduction in potential for immunogenicity, upon administration to a human subject, of a chimeric receptor identical to a receptor having this region, but containing the modification or combination of modifications in this region.

Example 3: Comparison of *In Silico* Analysis and *In Vitro* Binding of Peptides Derived from Junction Regions of a CAR for Binding to HLA Class I

[0292] Actual binding affinities for certain HLA class I alleles (A*02:01, A*03:01, A*11:01, and B*08:01) were assessed *in vitro* for exemplary overlapping 9 amino acid peptide sequences within a portion of the 26 amino acid junction region spanning the CD28 and 4-1BB junction. Specifically, assessment was of a series of overlapping 9-mer peptides derived from

the sequence VAFIIFWVKRGRKKLL (set forth in SEQ ID NO: 7), which contains a portion of the CD28 transmembrane domain and 4-1BB costimulatory domain spanning the junction between the domains (bond joining the two amino acids noted in underline). In addition, a series of overlapping 9-mer peptides of each of a number of different variants of this portion also were assessed, each variant containing a mutation or mutations in this region as described in Example 2.

[0293] The various 9-mer overlapping peptides were synthesized and their purity tested by MALDI-TOF Mass Spectrometry. The synthetic peptides were then incubated with recombinant MHC molecules to assess binding properties using the REVEAL Epitope Discovery System, which is a high-throughput binding assay that measures the degree to which each peptide is able to stabilize a ternary MHC-peptide complex (ProImmune, Oxford, United Kingdom). Each peptide was separately tested for this ability with respect to each of the HLA class I alleles, normalized to the degree observed for a positive control (known T cell epitope for the relevant allele). The results are reported as a score, in which the binding was normalized to the positive control peptide set at 100%. In this analysis, a score of greater than 50 generally was considered to represent good or high affinity binding.

[0294] The results were compared to predicted binding (IC₅₀) values obtained for binding of the same peptide:MHC complex, using the *in silico* prediction methods as described in Example 2. Since the maximum IC₅₀ value predicted was about 50,000, the IC₅₀ values were log transformed, subtracted from LOG(50000) and divided by LOG(50000) to obtain a normalized *in silico* score ((Log(50000) – logIC₅₀) / Log(50000)). In this analysis, an *in silico* binding prediction score of greater than 2.0 generally was considered to represent predicted good or high affinity binding.

[0295] The results are set forth in Table 2A (HLA-A*02:01 and HLA-A*03:01) and Table 2B (HLA-A*11:01 and HLA-B*08:01). In general, the *in silico* binding predictions were predictive of the actual *in vitro* binding results. In some cases, a relatively higher binding was predicted *in silico*, but not observed in the *in vitro* assay.

[0296] The results also were consistent with predicted binding affinity being generally predictive of affinity as measured in the *in vitro* assay. Additionally, the results demonstrated successful reduction of binding affinity to an HLA by modifications within a junction region, and that it was possible to modify the sequence in a way that resulted in a lower predicted or actual binding affinity or score of one of the overlapping potential epitopes, without increasing

(or while also reducing) binding affinity or score for another of the overlapping epitopes containing the same residue. In some embodiments, such mutations or modifications may be particularly advantageous. As a non-limiting example of the results, modifications K28L, R31H, L34S and/or L34A, with reference to numbering set forth in SEQ ID NO:5, generally resulted in a reduced predicted or actual binding affinity or score for at least one HLA allele and/or for at least one peptide within the region assessed, without resulting in a higher binding affinity to another HLA allele and/or without resulting in a higher binding affinity for another peptide.

Table 2A: In Silico and In Vitro MHC binding of Variant Peptides

Over-lapping Reference Peptide	Peptide Sequence	Mutation	A*02:01			A*03:01			SEQ ID NO.
			IEDB IC50	In Silico Score	In Vitro Score	IEDB IC50	In Silico	In Vitro	
1	VAFIIFWVK	none	19617	0.41	0.70	262	2.28	1.50	16
2	AFIIFWVKR	none	25314	0.30	0.50	17846	0.45	5.70	17
3	FIIFWVKRG	none	7967	0.80	10.10	23693	0.32	1.40	18
4	IIFWVKRGR	none	24769	0.31	1.70	406	2.09	24.30	19
5	IFWVKRGRK	none	30463	0.22	4.50	3482	1.16	20.50	20
6	FWVKRGRKK	none	28878	0.24	3.50	17327	0.46	2.20	21
7	WVKRGRKKL	none	27956	0.25	1.40	22961	0.34	13.10	22
1	VAFIIFWVS	K28S	12273	0.61	70.60	22660	0.34	0.70	23
2	AFIIFWVSR	K28S	23924	0.32	4.50	18157	0.44	0.30	24
3	FIIFWVSRG	K28S	3382	1.17	0.20	21751	0.36	0.10	25
4	IIFWVSRGR	K28S	21442	0.37	2.60	155	2.51	25.30	26
5	IFWVSRGRK	K28S	30615	0.21	1.30	1880	1.42	39.20	27
6	FWVSRGRKK	K28S	28679	0.24	1.50	17832	0.45	2.90	28
7	WVSRGRKKL	K28S	23551	0.33	2.30	22394	0.35	2.10	29
1	VAFIIFWVL	K28L	1336	1.57	0.20	20145	0.39	0.00	30
2	AFIIFWVLR	K28L	22444	0.35	5.70	15583	0.51	0.30	31
3	FIIFWVLRG	K28L	2037	1.39	7.20	20853	0.38	0.10	32
4	IIFWVLRGR	K28L	17613	0.45	8.40	238	2.32	2.80	33
5	IFWVLRGRK	K28L	30293	0.22	2.90	3675	1.13	19.50	34
6	FWVLRGRKK	K28L	28857	0.24	3.30	16996	0.47	0.40	35
7	WVLRGRKKL	K28L	19522	0.41	2.40	23063	0.34	0.70	36
1	VAFIIFWVH	K28H	23252	0.33	3.10	10359	0.68	0.30	37
2	AFIIFWVHR	K28H	22819	0.34	0.30	18506	0.43	0.30	38
3	FIIFWVHRG	K28H	1691	1.47	37.30	22930	0.34	0.00	39
4	IIFWVHRGR	K28H	23573	0.33	4.70	326	2.19	30.10	40
5	IFWVHRGRK	K28H	29930	0.22	7.10	1062	1.67	37.80	41
6	FWVHRGRKK	K28H	28189	0.25	1.60	17052	0.47	3.60	42
7	WVHRGRKKL	K28H	25035	0.30	1.60	22278	0.35	3.40	43
1	VAFIIFWVA	K28A	2733	1.26	28.50	21010	0.38	0.00	44

2	AFIIFWVAR	K28A	23072	0.34	2.20	17755	0.45	0.50	45
3	FIIFWVARG	K28A	1902	1.42		22486	0.35		102
4	IIFWVARGR	K28A	22077	0.36	9.60	206	2.39	18.60	46
5	IFWVARGRK	K28A	30251	0.22	1.00	3893	1.11	53.70	47
6	FWVARGRKK	K28A	28353	0.25	13.50	16210	0.49	5.00	48
7	WVARGRKKL	K28A	22630	0.34	8.80	22548	0.35	1.30	49
1	VAFIIFWVQ	K28Q	17921	0.45	5.30	22927	0.34	0.20	50
2	AFIIFWVQR	K28Q	24165	0.32	0.40	19279	0.41	0.20	51
3	FIIFWVQRG	K28Q	4152	1.08	16.80	23136	0.33	0.40	52
4	IIFWVQRGR	K28Q	21783	0.36	5.80	231	2.34	18.00	53
5	IFWVQRGRK	K28Q	30704	0.21	6.10	3177	1.20	36.70	54
6	FWVQRGRKK	K28Q	29501	0.23	5.20	18619	0.43	6.40	55
7	WVQRGRKKL	K28Q	24480	0.31	8.10	23630	0.33	4.90	56
4	IIFWVKRGS	R31S	18902	0.42	13.60	19450	0.41	16.20	57
5	IFWVKRGSK	R31S	29391	0.23	2.40	3348	1.17	10.40	58
6	FWVKRGSKK	R31S	28317	0.25	1.90	14227	0.55	5.90	59
7	WVKRGSKKL	R31S	23485	0.33	3.00	22637	0.34	1.90	60
4	IIFWVKRGL	R31L	2692	1.27	82.20	16661	0.48	15.90	61
5	IFWVKRGLK	R31L	29250	0.23	2.40	1973	1.40	26.90	62
6	FWVKRGLKK	R31L	27554	0.26	9.40	14434	0.54	2.70	63
7	WVKRGLKKL	R31L	20709	0.38	24.50	22985	0.34	10.00	64
4	IIFWVKRGH	R31H	27453	0.26	5.30	1665	1.48	26.60	65
5	IFWVKRGHK	R31H	29806	0.22	2.20	3806	1.12	9.00	66
6	FWVKRGHKK	R31H	27689	0.26	2.60	16743	0.48	4.10	67
7	WVKRGHKKL	R31H	25923	0.29	3.90	22313	0.35	1.40	68
4	IIFWVKRGA	R31A	6107	0.91	85.90	16069	0.49	42.90	69
5	IFWVKRGAK	R31A	29354	0.23	13.80	3470	1.16	22.00	70
6	FWVKRGAKK	R31A	28151	0.25	4.30	17066	0.47	4.70	71
7	WVKRGAKKL	R31A	24746	0.31	4.70	22982	0.34	4.10	72
4	IIFWVKRGN	R31N	25979	0.28	9.30	18552	0.43	4.30	73
5	IFWVKRGNK	R31N	29978	0.22	3.50	2669	1.27	8.00	74
6	FWVKRGNKK	R31N	28430	0.25	8.70	17713	0.45	3.60	75
7	WVKRGNKKL	R31N	24790	0.30	2.40	22813	0.34	0.90	76
4	IIFWVQRGS	K28Q/R31S	14326	0.54	22.40	17741	0.45	4.50	77
5	IFWVQRGSK	K28Q/R31S	29540	0.23	33.50	3052	1.21	20.30	78
6	FWVQRGSKK	K28Q/R31S	28907	0.24	0.80	15992	0.50	1.90	79
7	WVQRGSKKL	K28Q/R31S	18121	0.44	4.40	23279	0.33	1.20	80
4	IIFWVQRGA	K28Q/R31A	2658	1.27	95.30	13467	0.57	2.70	81
5	IFWVQRGAK	K28Q/R31A	29482	0.23	11.70	3205	1.19	17.00	82
6	FWVQRGAKK	K28Q/R31A	28792	0.24	2.20	18649	0.43	2.70	83

7	WVQRGAKKL	K28Q/R3 1A	20217	0.39	3.00	23651	0.33	2.00	84
4	IIFWVQRGN	K28Q/R3 1N	23103	0.34	13.00	16590	0.48	3.60	85
5	IFWVQRGNK	K28Q/R3 1N	30164	0.22	16.10	2438	1.31	23.20	86
6	FWVQRGNKK	K28Q/R3 1N	29113	0.23	3.10	19165	0.42	1.30	87
7	WVQRGNKKL	K28Q/R3 1N	19790	0.40	4.30	23457	0.33	1.50	88
7	WVKRGRKKS	L34S	30812	0.21	3.90	25365	0.29	0.90	89
7	WVKRGRKKA	L34A	28556	0.24	4.50	24086	0.32	0.90	90
7	WVQRGNKKKS	K28Q/L3 4S	26883	0.27	1.20	25680	0.29	0.70	91
7	WVQRGNKKKA	K28Q/L3 4A	21998	0.36	1.90	24564	0.31	1.20	92
1	VAFIIFWVR	K28R	20045	0.40	1.20	5746	0.94	0.70	100
2	AFIIFWVRR	K28R	25059	0.30	1.40	17924	1.00		101

Table 2B: In Silico and In Vitro MHC binding of Variant Peptides

Overlapping Reference Peptide	Peptide Sequence	Mutation	A*11:01			B*08:01			SEQ ID NO.
			IEDB IC50	In Silico Score	In Vitro Score	IEDB IC50	In Silico Score	In Vitro Score	
1	VAFIIFWVK	wt	19	3.42	109.20	15806	0.50	0.30	16
2	AFIIFWVKR	wt	3271	1.18	25.90	23244	0.33	1.90	17
3	FIIFWVKRG	wt	22946	0.34	0.40	18081	0.44	0.10	18
4	IIFWVKRGR	wt	706	1.85	54.50	23025	0.34	0.00	19
5	IFWVKRGRK	wt	9701	0.71	29.30	23513	0.33	0.40	20
6	FWVKRGRKK	wt	22175	0.35	6.50	21764	0.36	0.00	21
7	WVKRGRKKL	wt	24020	0.32	4.80	225	2.35	28.50	22
1	VAFIIFWVS	K28S	12403	0.61	25.10	13796	0.56	0.10	23
2	AFIIFWVSR	K28S	2175	1.36	54.00	21862	0.36	0.30	24
3	FIIFWVSRG	K28S	20651	0.38	5.10	17854	0.45	0.40	25
4	IIFWVSRGR	K28S	162	2.49	68.90	23445	0.33	0.00	26
5	IFWVSRGRK	K28S	5778	0.94	39.20	23448	0.33	0.00	27
6	FWVSRGRKK	K28S	21126	0.37	6.30	23067	0.34	0.00	28
7	WVSRGRKKL	K28S	23684	0.32	4.40	3208	1.19	6.20	29
1	VAFIIFWVL	K28L	14191	0.55	0.20	1196	1.62	0.00	30
2	AFIIFWVLR	K28L	850	1.77	17.20	23012	0.34	0.20	31
3	FIIFWVLRG	K28L	19162	0.42	2.40	17427	0.46	0.20	32
4	IIFWVLRGR	K28L	185	2.43	35.20	23298	0.33	0.40	33
5	IFWVLRGRK	K28L	6386	0.89	29.30	23324	0.33	0.00	34
6	FWVLRGRKK	K28L	21862	0.36	5.00	23437	0.33	0.00	35
7	WVLRGRKKL	K28L	23725	0.32	3.20	1342	1.57	4.00	36

1	VAFIIFWV H	K28H	1209	1.62	12.30	16202	0.49	0.00	37
2	AFIIFWV H R	K28H	4939	1.01	34.90	22682	0.34	0.30	38
3	FIIFWV H R G	K28H	19850	0.40	0.90	17464	0.46	0.20	39
4	IIFWV H R G R	K28H	196	2.41	77.50	22662	0.34	0.20	40
5	IFWV H R G R K	K28H	7133	0.85	36.60	22030	0.36	0.10	41
6	FWV H R G R K K	K28H	22214	0.35	5.60	23778	0.32	0.00	42
7	WV H R G R K K L	K28H	23844	0.32	5.60	814	1.79	19.20	43
1	VAFIIFWV A	K28A	12499	0.60	2.80	5131	0.99	0.00	44
2	AFIIFWV A R	K28A	2784	1.25	51.80	21850	0.36	0.20	45
3	FIIFWV A R G	K28A	20922	0.38		18463	0.43		102
4	IIFWV A R G R	K28A	239	2.32	70.60	23580	0.33	0.30	46
5	IFWV A R G R K	K28A	8772	0.76	34.90	23612	0.33	0.00	47
6	FWV A R G R K K	K28A	20762	0.38	8.90	23035	0.34	0.00	48
7	WV A R G R K K L	K28A	23920	0.32	13.80	2821	1.25	32.40	49
1	VAFIIFWV Q	K28Q	15477	0.51	3.90	14875	0.53	0.20	50
2	AFIIFWV Q R	K28Q	2174	1.36	15.00	23016	0.34	0.00	51
3	FIIFWV Q R G	K28Q	22161	0.35	1.50	17653	0.45	0.60	52
4	IIFWV Q R G R	K28Q	361	2.14	72.80	23548	0.33	0.10	53
5	IFWV Q R G R K	K28Q	9561	0.72	128.50	23670	0.32	0.00	54
6	FWV Q R G R K K	K28Q	22394	0.35	5.70	22604	0.34	1.90	55
7	WV Q R G R K K L	K28Q	23688	0.32	12.80	2512	1.30	45.10	56
4	IIFWV K R G S	R31S	18923	0.42	100.00	22325	0.35	0.40	57
5	IFWV K R G S K	R31S	7476	0.83	47.90	21691	0.36	1.20	58
6	FWV K R G S K K	R31S	19910	0.40	5.60	20823	0.38	0.10	59
7	WV K R G S K K L	R31S	24090	0.32	11.80	585	1.93	56.10	60
4	IIFWV K R G L	R31L	20182	0.39	32.40	17368	0.46	1.70	61
5	IFWV K R G L K	R31L	4201	1.08	61.10	23537	0.33	58.30	62
6	FWV K R G L K K	R31L	17309	0.46	25.30	20839	0.38	6.30	63
7	WV K R G L K K L	R31L	24095	0.32	22.10	765	1.82	65.70	64
4	IIFWV K R G H	R31H	7117	0.85	19.50	23227	0.33	0.10	65
5	IFWV K R G H K	R31H	10783	0.67	30.90	23461	0.33	3.20	66
6	FWV K R G H K K	R31H	17635	0.45	27.60	20754	0.38	0.40	67
7	WV K R G H K K L	R31H	23924	0.32	2.80	269	2.27	47.60	68
4	IIFWV K R G A	R31A	19134	0.42	22.60	19585	0.41	0.90	69
5	IFWV K R G A K	R31A	8311	0.78	69.70	21592	0.36	2.20	70
6	FWV K R G A K K	R31A	20234	0.39	13.20	21762	0.36	0.30	71
7	WV K R G A K K L	R31A	23857	0.32	4.60	1366	1.56	34.10	72
4	IIFWV K R G N	R31N	19351	0.41	42.10	22864	0.34	0.90	73
5	IFWV K R G N K	R31N	6780	0.87	45.90	24238	0.31	0.10	74
6	FWV K R G N K K	R31N	20732	0.38	7.30	21565	0.37	0.10	75
7	WV K R G N K K L	R31N	24036	0.32	4.10	1181	1.63	65.90	76
4	IIFWV Q R G S	K28Q/R31S	18031	0.44	16.90	22961	0.34	0.10	77
5	IFWV Q R G S K	K28Q/R31S	7300	0.84	103.30	22846	0.34	0.00	78
6	FWV Q R G S K K	K28Q/R31S	20419	0.39	6.80	21853	0.36	0.00	79
7	WV Q R G S K K L	K28Q/R31S	23740	0.32	5.90	5230	0.98	6.80	80

4	IIFWVQRGA	K28Q/R31A	18055	0.44	56.50	20759	0.38	0.20	81
5	IFWVQRGAK	K28Q/R31A	8237	0.78	61.30	22801	0.34	0.20	82
6	FWVQRGAKK	K28Q/R31A	20696	0.38	18.30	22612	0.34	0.00	83
7	WVQRGAKKL	K28Q/R31A	23552	0.33	3.80	8314	0.78	11.40	84
4	IIFWVQRGN	K28Q/R31N	18437	0.43	29.00	23425	0.33	0.00	85
5	IFWVQRGNK	K28Q/R31N	6566	0.88	67.50	24059	0.32	0.10	86
6	FWVQRGNKK	K28Q/R31N	21228	0.37	5.90	22436	0.35	0.00	87
7	WVQRGNKKL	K28Q/R31N	23751	0.32	10.70	7869	0.80	32.00	88
7	WVKRGRKKS	L34S	23906	0.32	6.70	10345	0.68	4.30	89
7	WVKRGRKKA	L34A	23864	0.32	3.10	1225	1.61	3.10	90
7	WVQRGNKKS	K28Q/L34S	23612	0.33	5.00	21235	0.37	0.00	91
7	WVQRGNKKA	K28Q/L34A	23576	0.33	0.80	14247	0.55	0.70	92
1	VAFIIFWVR	K28R	108	2.67	34.60	16290	0.49	0.10	100
2	AFIIFWVR	K28R	3387	1.17	1.50	22717	0.34	0.30	101

Example 4: Analysis of Peptides Derived from Junction Region of a CAR for Binding to HLA-A2:01

[0297] In order to identify CAR-derived peptides potentially capable of inducing immunogenic responses, a series of overlapping peptides within the non-variant (reference) sequence containing the junction between the CD28 transmembrane domain and 4-1BB costimulatory domain of a CAR were assessed *in silico*. Algorithms were used to predict binding affinities for the peptide groove of a common human MHC class I molecule (HLA-A2:01) using *in silico* analysis to predict affinity for binding. As set forth in FIG. 3, the assessed portion of the CAR had the sequence CYSLLVTVAFIIFWVKRGRKKLLYIFKQPF (set forth in SEQ ID NO: 6), which contains a portion of the of the CD28 transmembrane domain (set forth in SEQ ID NO:2) and a portion of the 4-1BB costimulatory domain (set forth in SEQ ID NO:3), with the residues spanning the junction of the domains shown by underline. Predicted HLA-A2:01 binding affinity was assessed *in silico* for a series of 140 overlapping peptides of 8-14 amino acids of the sequence set forth in SEQ ID NO:6. Thirty-five (35) of the peptides contained only sequence from the transmembrane domain portion; 35 of the peptides contained only from the costimulatory domain portion, and 70 of the peptides had a junction or fusion region sequence, containing amino acid residues bridging the junction between the domains. For this assessment, peptide fragments predicted to bind to HLA-A2:01 with a dissociation constant of 0 nM to 50 nM were considered predicted to bind with high affinity. Peptide fragments predicted to bind with a dissociation constant of 51 nM to 1000 nM were considered predicted to bind with low

affinity. Peptide fragments predicted to bind with a predicted affinity of 1000 nM to 5000 nM were considered predicted to bind with rare affinity. The results are presented in FIG. 3.

[0298] As shown in FIG. 3, two of the peptides derived from the reference sequence in this region, each containing a sequence with an overlapping region spanning the junction between the domains were predicted to exhibit low binding affinity for HLA-A2:01. Specifically, a 14-mer peptide having the sequence FIIFWVKRGRKKLL (SEQ ID NO: 10), was predicted to bind with a dissociation constant of 294 nM, and a 13-mer peptide having the sequence of FIIFWVKRGRKKL (SEQ ID NO: 11) was predicted to bind with a dissociation constant of 618 nM. These peptides each included a portion of the 15-mer peptide set forth in SEQ ID NO:8 and identified in Example 1. Shorter 8-mer to 12-mer peptides within this sequence were not predicted to exhibit binding to HLA-A2:01. Another 13-mer peptide containing the amino acid sequence IIFWVKRGRKKLL (SEQ ID NO: 12) was predicted to have a rare binding affinity with a predicted dissociation constant of approximately 3000 nM. None of the remaining fragments that bridged the junction between the two domains were predicted by this assay to exhibit binding affinity for HLA-A2:01 (all had a predicted dissociation constant of far greater than 5000 nM, and in most cases higher than 14,000 nm or 20,000 nM or greater). In each of the peptides predicted to bind to HLA-A2:01, neither of the two junction-spanning residues (VK) themselves was predicted to be an anchor residue; rather, such peptides contained these residues in non-flanking positions.

[0299] Approximately 15 of the peptides containing sequence derived only from the transmembrane domain were predicted to have a dissociation constant for HLA-A2:01 of less than 5000 nM. Two peptides containing sequence only from the co-stimulatory domain were predicted to have a dissociation constant for HLA-A2:01 binding of less than 5000 nM. The costimulatory domain and transmembrane domain in the assessed sequence are derived from endogenous human sequences, which generally are less likely to be immunogenic to a human subject. For example, in the study described in Example 1, no immune responses were detected that were specific for peptide sequences solely within either one of these domains of the CAR. Accordingly, variants of peptides containing sequence spanning the junction region were assessed.

[0300] To generate variant peptides predicted to have reduced binding affinities to HLA-A2:01 and/or reduced immunogenicity in a human subject having this HLA allele, a variant sequence was generated *in silico*, containing mutations in the junction region as compared to the

sequence set forth in SEQ ID NO:6. Given that peptides containing the junction-spanning “VK” residues (at non-anchor positions) were predicted to exhibit high binding affinities for HLA-A2:01, two asparagine residues were inserted in the junction between the CD28 transmembrane and 4-1BB co-stimulatory domains. The variant contained the sequence CYSLLVTVAFIIFWVNNKRGRKKLLYIFKQPF (set forth in SEQ ID NO: 13, the sequence flanking the junction that was generated by insertion of the asparagine residues is shown in underline). The exemplary variant sequence of SEQ ID NO: 13 was assessed by the same predictive methods. To assess predicted binding affinities for this variant sequence, a series of 154 overlapping fragments of 8-14 amino acids of the sequence set forth in SEQ ID NO: 13 were assessed by *in silico* analysis as described above, whereby 35 peptides had a sequence only in the transmembrane portion, 35 peptides had a sequence only in the costimulatory domain portion and 84 peptides contained a junction region sequence containing amino acids bridging the domains, including one or both of the inserted asparagine residues.

[0301] The results are depicted in FIG. 3. As shown, overall, the HLA-A2:01 binding affinities of overlapping peptides within the variant region containing the junction, collectively, were substantially reduced as compared to the non-variant sequence. In particular, the predicted dissociation constant for binding to HLA-A2:01 of peptides in the portion of the junction region previously predicted to be immunogenic was substantially reduced. For example, peptide variants IIFWVNNKRGRKKL (SEQ ID NO: 14) and IIFWVNNKRGRKK (SEQ ID NO: 15), which included asparagine residues in the altered region flanking the junction compared to peptides identified as set forth in SEQ ID NOS:10 and 11, respectively, were predicted to exhibit no detectable binding affinity to HLA-A2:01. Two 14-mer peptides, FIIFWVNNKRGRKK (SEQ ID NO:96) and IFWVNNKRGRKKLL (SEQ ID NO:97), were predicted to exhibit a dissociation constant for binding to this HLA indicating a rare binding affinity, within the range of 1000 nM to 5000 nM. All other peptides containing the modified junction region sequence were predicted to exhibit a dissociation constant of greater than 5000 nM, and in most cases higher than 14,000 nM or 20,000 nM or greater, and thus were not predicted to exhibit binding affinity for HLA-A2:01 by this assessment. Additionally, the modification of the junction region sequence did not create any new peptides predicted to have higher binding affinities for HLA-A2:01 within the costimulatory or transmembrane domain regions.

Example 5: Administration of anti-CD22 CAR-Expressing Cells to Subjects Previously Treated with anti-CD19 CAR

[0302] Six subjects with relapsed/refractory CD22⁺ B cell acute lymphoblastic leukemia (ALL) were administered autologous T cells expressing an anti-CD22 chimeric antigen receptor (CAR). The CAR included a human anti- CD22 scFv antibody, a CD8alpha transmembrane domain, a 4-1BB intracellular signaling domain, and a CD3zeta intracellular signaling domain.

[0303] All subjects had previously undergone at least one prior allogeneic hematopoietic stem cell transplant and had received treatment with one of various CD19-directed CAR-T cell therapies. Five of the subjects had relapsed with ALL on which CD19 was not detected (“CD19 neg”) and one subject was otherwise a non-responder to the prior CD19 CAR therapy.

[0304] Table 3 summarizes the characteristics of the treated patients.

Table 3: Patient Characteristics						
ID	Age/Sex	Prior HCT	Prior anti-CD19 CAR	CD19 neg relapse	CD22 site density	Pre-HCT disease burden (% leukemia in aspirate)
1	22/M	Y	Y	Y	2084	>95%
2	20/F	Y (2)	Y	Y	13452	5%
3	22/M	Y	Y	Y	846	>90%
4	22/M	Y	Y	N	2589	95%
5	7/F	Y	Y	Y	2839	32%
6	17/F	Y	Y	Y	2185	1%

HCT: hematopoietic cell transplantation.

[0305] Prior to administration of the cells, patients underwent autologous leukapheresis to harvest peripheral blood mononuclear cells (PBMCs). T cells were isolated from the harvested PBMCs by immunoaffinity-based enrichment for CD3 expression and cultured in the presence of anti-CD3/-CD28 beads, followed by transduction with a lentiviral vector encoding the anti-CD22 CAR. The cells were cultured for 7-10 days. Subjects received induction chemotherapy with 25 mg/m² fludarabine on Days -4, -3 and -2 and 900 mg/m² cyclophosphamide on day -2 (cell infusion on Day 0). Each patient received an initial CAR T cell dose of 3 x 10⁵ transduced T-cells/recipient weight (kg) by intravenous infusion. The second subject enrolled developed grade 3 diarrhea, meeting the criteria for dose-limiting toxicity (DLT), which led to dose expansion at the first dose-level to treat a total of 6 subjects. No subsequent DLTs were seen at this dosage. Two subjects developed grade 1 cytokine release syndrome (CRS), one subject developed grade 2 CRS, and in two subjects CRS was not present.

[0306] The number of CAR-T cells in peripheral blood, bone marrow or cerebrospinal fluid was determined at certain timepoints post-treatment by incubating cells with CD22-Fc. For patients in which expansion was observed, evidence for CAR-T cell expansion was seen in peripheral blood, bone marrow and cerebrospinal fluid, beginning at about day 7. The maximum or peak CAR-T cell expansion was generally observed between about day 12 and about day 15 post-infusion. Table 7 sets forth the maximum or peak percentage of anti-CD22 CAR-T cells observed in this assessment period as a percentage of total T cells in each sample for the treated subjects. Clinical responses were evaluated at day 28 (+/- 4 days) post-infusion.

[0307] As shown in Table 4, the results were consistent with responses being generally correlated to degree of CAR-T cell expansion. For three subjects that exhibited no or low CAR-T cell expansion also showed evidence of disease progression. Two other subjects had stable disease, and one was observed with complete remission with no MRD. Flow cytometric CAR persistence was detected out to 47 days post-infusion in this subject, with remission maintained for 3 months post-infusion. The results demonstrate safe, feasible, and clinically active anti-CD22 CAR T-cell therapy in subjects having undergone (and having become non-responsive to, e.g., due to epitope/antigen loss) previous anti-CD19 CAR therapy.

Table 4: Treatment response

ID	Maximum CAR expansion (flow)			CRS	Best Response
	PB	Marrow	CSF		
1	0	0	n/a	None	PD
2	52.3%	19.5%	0%	Gr 1	MRD neg CR
3	73%	36%	32%	Gr 1	SD
4	6%	1%	0%	Gr 2	SD
5	0%	1.3%	0%	None	PD
6	1.8%	2%	0%	None	PD

PB: peripheral blood; CSF: cerebrospinal fluid; CRS: cytokine release syndrome; PD: progressive disease; MRD: minimal residual disease; CR: complete remission; SD: stable disease.

Example 6: Comparison of *In Silico* Analysis and *In Vitro* Binding of Peptides Derived from Junction Regions of a CAR for Binding to HLA Class II

[0308] Actual binding affinities for certain HLA class II alleles (DPA1*01:03;DPB1*04:01, DRA*01:01;DRB1*03:01, DRA*01:01, DRB1*15:01, DRA*01:01;DRB1*11:01, DPA1*01:03;DPB1*04:02, DPA1*01:03;DPB1*03:01, DPA1*01:03;DPB1*01:01, DPA1*02:01;DPB1*01:01, DPA1*02:01;DPB1*04:02, DRA*01:01, DRB1*11:04, DRA*01:01, DRB1*01:02, and DPA1*02:01;DPB1*15:01) were assessed *in vitro* for

exemplary overlapping 15 amino acid peptide sequences within a portion of the region spanning the junction between the CD28 and 4-1BB-derived sequences of the CAR described in Example 4. Specifically, assessment was of a series of overlapping 15-mer peptides derived from the sequence CYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQT (set forth in SEQ ID NO: 160), which contains a portion of the CD28-derived transmembrane domain and 4-1BB-derived costimulatory domain and spans the junction between the domains (bond joining the two amino acids noted in underline). In addition, a series of overlapping 15-mer peptides of each of a number of different variants of this portion also were assessed, each variant containing a mutation or mutations in this region as described in Example 2.

[0309] The various 15-mer overlapping peptides were synthesized and their purity tested by MALDI-TOF Mass Spectrometry. The synthetic peptides were then incubated with recombinant MHC molecules to assess binding properties using the REVEAL Epitope Discovery System as described in Example 3. Each peptide was separately tested for this ability with respect to each of the HLA class II alleles, normalized to the degree observed for a positive control (known T cell epitope for the relevant allele). A score was calculated for each allele, in which the binding was normalized to the positive control peptide set at 100%. The potential impact in the population based on these scores was calculated as the sum of the individual allele scores for each peptide.

[0310] The results are set forth in FIG. 7. As shown, the results demonstrated that modifications within the junction region resulted in successful reduction for HLA class II binding of at least one peptide within the region.

[0311] For overlapping 15-mer peptides present within the native junction region, the results were compared to predicted binding (IC₅₀) values obtained for binding of the same peptide:MHC complex, using the *in silico* prediction methods as described in Example 2. Since the maximum IC₅₀ value predicted was about 50,000, the IC₅₀ values were log transformed, subtracted from LOG(50000) and divided by LOG(50000) to obtain a normalized *in silico* score ((Log(50000) – logIC₅₀) / Log(50000)). Immunogenicity scores (weighted based on relative frequency in the population of the individual HLA class II alleles) were obtained for the normalized *in silico* and actual binding affinities. The results are set forth in FIG. 8. In general, the *in silico* binding predictions were predictive of, and in some cases were over-predictive of, the actual *in vitro* binding results.

Example 7: Engineering and Characterization of T Cells Transduced with CARs Having Modified Junction Regions

[0312] CAR-engineered T cells were generated by transduction of primary T cells with a lentiviral vector encoding either (1) the original chimeric antigen receptor (CAR) as described in Example 1, containing an anti-CD19 scFv, a hinge domain, the junction region set forth in SEQ ID NO:5 (including a transmembrane domain derived from the native CD28 and an intracellular signaling domain derived from the native 4-1BB) (deemed the “native” junction region) and a CD3-zeta intracellular signaling domain or (2) one of a number of particular variants thereof, individually containing a modified junction region in which one or more mutations were introduced in the junction region spanning the junction between the CD28 transmembrane domain and 4-1BB costimulatory signaling domain. Each of the particular variant CARs contained a modified junction region having a mutation (with reference to SEQ ID NO:5) selected from among: K28Q/R31N/L34S, K28Q/R31N/L34A, R31N/L34S, R31N/L34A, K28Q/L34A, K28Q/R31S, K28Q/R31N, K28Q/R31A, L34S, L34A, R31S, R31N, R31A, K28S, K28Q, K28L, K28H, and K28A.

[0313] Primary human CD4⁺ and CD8⁺ T cells were isolated by immunoaffinity-based selection from human PBMC samples obtained from healthy donors. The resulting cells were stimulated by culturing with an anti-CD3/anti-CD28 reagent prior to engineering with the respective CAR. Cells were transduced using a lentiviral vector containing a nucleic acid molecule encoding the CAR and a nucleic acid encoding a truncated EGFR (EGFRt), for use as a surrogate marker for transduction, separated by a sequence encoding a T2A ribosome switch. A mock transduction was used as negative control. Transduced cells were used for expression analysis and cytolytic activity assay.

A. CAR Expression

[0314] Cell surface CAR expression (as indicated via the surrogate marker) was assessed at day 17 post-transduction with nucleic acid encoding the native CAR or each of the modified CARs. Cells were stained with anti-EGFR antibody to detect EGFRt as a surrogate to verify CAR expression and assessed by flow cytometry. CAR expression was similar in cells transduced to express the various variant CARs containing a modified junction region, and was comparable to expression of the CAR containing the native junction region.

B. Cytolytic Activity

[0315] Cells engineered to express the native or variant CARs were assessed for cytolytic activity against K562 target cells expressing the CD19 antigen (K562-CD19). The T cells were incubated with the target cells (K562-CD19) at various effector:target ratios (CAR:Target ratios of 1:1, 2:1 and 4:1) in a well of a culture plate. Lytic activity of the engineered T cells was assessed by measuring the number of caspase-positive target cells per well. The results showed that in this study, the T cells expressing the various variant CARs containing the modified junction region were able to kill CD19-expressing target cells in a target-specific manner, to a similar degree as T cells expressing the CAR containing the “native” junction region.

Example 8: Assessment of Surface Expression and Functionality of CARs Having Modified Junction Regions

[0316] CAR-engineered T cells were generated by transduction of primary T cells with a nucleic acid encoding either (1) the original chimeric antigen receptor (CAR) described in Example 1 containing an anti-CD19 scFv, a hinge domain, the native junction region set forth in SEQ ID NO:5 (including a transmembrane domain derived from the native CD28 and an intracellular signaling domain derived from the native 4-1BB), or (2) a variant thereof containing a modified junction region having a mutation selected from among: K28L, R31H, L34A, or L34S with reference to SEQ ID NO:5. Cell surface CAR expression and functional properties of T cells engineered with CARs containing a modified junction region were assessed.

A. Surface Expression

[0317] To directly assess surface expression of the CARs, CAR-engineered T cells were stained with an anti-idiotypic antibody specific for the anti-CD19 scFv contained in the CAR. The engineered cells also were assessed for surface expression of the surrogate marker EGFRt using an anti-EGFR antibody. To detect intracellular expression of the CARs, cells were first permeabilized and then stained under similar conditions using the anti-idiotypic antibody and anti-EGFR antibody. Expression of the CAR and surrogate marker was analyzed by flow cytometry in CD4+ or CD8+ subsets.

[0318] FIG. 9A and 9B show that the variant CARs containing a modified junction region were expressed on the surface and intracellularly, respectively, in engineered CD4+ T cells. The level of surface expression of the variant CARs was similar or greater than the surface

expression of the unmodified CAR containing the native junction region. The surrogate marker, which was co-transduced with the CARs, was expressed similarly in all engineered cells.

[0319] The mean fluorescence intensity (MFI) of CAR surface expression, as determined using the CAR-specific anti-idiotypic antibody, was quantified in engineered T cells gated for CD4⁺ and CD8⁺ cells. As shown in FIG. 10, surface expression of the CARs was similar on both CD4⁺ and CD8⁺ T cells. Variant CARs containing a modified junction region having the L34A and L34S mutations exhibited increased surface expression as compared to the CAR with the native junction region.

B. Cytokine Expression

[0320] The production of cytokines by the anti-CD19 CAR T cells, expressing CARs containing either the native junction region or the various modified junction regions, was assessed. Engineered T cells were co-cultured with irradiated CD19-transduced K562 target cells (K562-CD19) or parental K562 cells not expressing the CD19 antigen in the presence of Golgi inhibitor. After stimulation, the cells were then fixed, permeabilized and intracellular IL-2 and IFN- γ cytokine levels were assessed by flow cytometry in CD4⁺/CAR⁺ cells and CD8⁺/CAR⁺ cells

[0321] Exemplary flow cytometry plots depicting intracellular levels of IL-2 and IFN- γ in CD4⁺/CAR⁺ cells and CD8⁺/CAR⁺ cells are shown in FIG. 11. As shown, a greater percentage of CD4⁺/CAR⁺ T cells and CD8⁺/CAR⁺ T cells expressing the L34A and L34S variant CARs exhibited intracellular expression of IL-2 after antigen stimulation as compared to cells engineered with the unmodified CAR. A greater percentage of CD8⁺/CAR⁺ T cells expressing the R31H and L34A CAR variants had increased IFN- γ cytokine expression after antigen stimulation as compared to cells engineered with the unmodified CAR. Without wishing to be bound by theory, the increased level of cytokine expression may roughly correlate with the higher surface expression observed for the R31H and L34A variant CARs.

C. CAR T Cell Expansion

[0322] The ability of cells to expand ex vivo following repeated stimulations in some aspects can indicate capacity of CAR-T cells to persist (e.g. following initial activation) and/or is indicative of function in vivo (Zhao et al. (2015) Cancer Cell, 28:415-28). CAR-T cells were generated as described above and cultured with irradiated target cells (K562-CD19). Cells were stimulated, harvested every 3-4 days and counted, and restimulated with new target cells using the same culture conditions after resetting cell number to initial seeding density for each round.

A total of 5 rounds of stimulation during a 17 day culture period were carried out. For each round of stimulation, the number of cells was determined.

[0323] As shown in FIG. 12, comparable initial growth of the anti-CD19 CAR-engineered cells was observed for cells expressing variant CARs containing a modified junction region as compared to cells expressing the CAR with the native junction region. After day 7 of restimulation, the degree of cell expansion declined in cells expressing the CAR containing the native junction region, whereas the variant CARs exhibited continued or similar expansion until at least day 10 of restimulation.

[0324] The present invention is not to be limited in scope by the embodiments disclosed herein, which are intended as single illustrations of individual aspects of the invention, and any that are functionally equivalent are within the scope of the invention. Various modifications to the models and methods of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and teachings, and are similarly intended to fall within the scope of the invention. Such modifications or other embodiments can be practiced without departing from the true scope and spirit of the invention.

SEQUENCES**Table 5: Sequences**

SEQ ID NO	Sequence	Note
1	ESKYGPPCPPCP	IgG4 hinge
2	FWVLVVVGVLACYSLLVTVAFIIFWV	CD28 transmembrane domain
3	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	4-1BB costimulatory domain (amino acids 214-255 of Q07011.1) Homo sapien
4	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR	CD3-zeta intracellular signaling domain
5	FWVLVVVGVLACYSLLVTVAFIIFWV <u>KRGRKKLL</u> YIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCEL	CD28-4-1BB
6	CYSLLVTVAFIIFWVKRGRKKLLYIFKQPF	Peptide
7	VAFIIFWVKRGRKKLL	Peptide
8	AFIIFWVKRGRKKLL	Peptide
9	FWVKRGRKKLLYIFK	Peptide
10	FIIFWVKRGRKKLL	Peptide
11	FIIFWVKRGRKKL	Peptide
12	IIFWVKRGRKKLL	Peptide
13	CYSLLVTVAFIIFWV <u>NN</u> KRGRKKLLYIFKQPF	Variant junction region
14	IIFWVNNKRGRKKL	Variant peptide
15	IIFWVNNKRGRKK	Variant peptide
16	VAFIIFWVK	Synthetic peptide
17	AFIIFWVKR	Synthetic peptide
18	FIIFWVKRG	Synthetic peptide
19	IIFWVKRGR	Synthetic peptide
20	IFWVKRGRK	Synthetic peptide
21	FWVKRGRKK	Synthetic peptide
22	WVKRGRKKL	Synthetic peptide
23	VAFIIFWVS	Synthetic peptide K28S
24	AFIIFWVSR	Synthetic peptide K28S
25	FIIFWVSRG	Synthetic peptide K28S
26	IIFWVSRGR	Synthetic peptide K28S
27	IFWVSRGRK	Synthetic peptide K28S
28	FWVSRGRKK	Synthetic peptide K28S
29	WVSRGRKKL	Synthetic peptide K28S
30	VAFIIFWVL	Synthetic peptide

		K28L
31	AFIIFWVLR	Synthetic peptide K28L
32	FIIFWVLRG	Synthetic peptide K28L
33	IIFWVLRGR	Synthetic peptide K28L
34	IFWVLRGRK	Synthetic peptide K28L
35	FWVLRGRKK	Synthetic peptide K28L
36	WVLRGRKKL	Synthetic peptide K28L
37	VAFIIFWVH	Synthetic peptide K28H
38	AFIIFWVHR	Synthetic peptide K28H
39	FIIFWVHRG	Synthetic peptide K28H
40	IIFWVHRGR	Synthetic peptide K28H
41	IFWVHRGRK	Synthetic peptide K28H
42	FWVHRGRKK	Synthetic peptide K28H
43	WVHRGRKKL	Synthetic peptide K28H
44	VAFIIFWVA	Synthetic peptide K28A
45	AFIIFWVAR	Synthetic peptide K28A
46	IIFWVARGR	Synthetic peptide K28A
47	IFWVARGRK	Synthetic peptide K28A
48	FWVARGRKK	Synthetic peptide K28A
49	WVARGRKKL	Synthetic peptide K28A
50	VAFIIFWVQ	Synthetic peptide K28Q
51	AFIIFWVQR	Synthetic peptide K28Q
52	FIIFWVQRG	Synthetic peptide K28Q
53	IIFWVQRGR	Synthetic peptide K28Q
54	IFWVQRGRK	Synthetic peptide K28Q
55	FWVQRGRKK	Synthetic peptide K28Q
56	WVQRGRKKL	Synthetic peptide

		K28Q
57	IIFWVKRGS	Synthetic peptide R31S
58	IFWVKRGSK	Synthetic peptide R31S
59	FWVKRGSKK	Synthetic peptide R31S
60	WVKRGSKKL	Synthetic peptide R31S
61	IIFWVKRGL	Synthetic peptide R31L
62	IFWVKRGLK	Synthetic peptide R31L
63	FWVKRGLKK	Synthetic peptide R31L
64	WVKRGLKKL	Synthetic peptide R31L
65	IIFWVKRGH	Synthetic peptide R31H
66	IFWVKRGHK	Synthetic peptide R31H
67	FWVKRGHKK	Synthetic peptide R31H
68	WVKRGHKKL	Synthetic peptide R31H
69	IIFWVKRGA	Synthetic peptide R31A
70	IFWVKRGAK	Synthetic peptide R31A
71	FWVKRGAKK	Synthetic peptide R31A
72	WVKRGAKKL	Synthetic peptide R31A
73	IIFWVKRGN	Synthetic peptide R31N
74	IFWVKRGNK	Synthetic peptide R31N
75	FWVKRGNKK	Synthetic peptide R31N
76	WVKRGNKKL	Synthetic peptide R31N
77	IIFWVQRGS	Synthetic peptide K28Q/R31S
78	IFWVQRGSK	Synthetic peptide K28Q/R31S
79	FWVQRGSKK	Synthetic peptide K28Q/R31S
80	WVQRGSKKL	Synthetic peptide K28Q/R31S
81	IIFWVQRGA	Synthetic peptide K28Q/R31A

82	IFWVQRGAK	Synthetic peptide K28Q/R31A
83	FWVQRGAKK	Synthetic peptide K28Q/R31A
84	WVQRGAKKL	Synthetic peptide K28Q/R31A
85	IIFWVQRGN	Synthetic peptide K28Q/R31N
86	IFWVQRGNK	Synthetic peptide K28Q/R31N
87	FWVQRGNKK	Synthetic peptide K28Q/R31N
88	WVQRGNKKL	Synthetic peptide K28Q/R31N
89	WVKRGRKKS	Synthetic peptide L34S
90	WVKRGRKKA	Synthetic peptide L34A
91	WVQRGNKKS	Synthetic peptide K28Q/L34S
92	WVQRGNKKA	Synthetic peptide K28Q/L34A
93	MGNSCYNIVATLLLVLNFERTSLQDPCSNCPAGTFCDNNRNQICSPCP PNSFSSAGGQRTCDICRQCKGVFTRTRKECSSTSNAECDCTPGFHCGLGAG CSMCEQDCKQGQELTKKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVL VNGTKERDVVCGPSPADLSPGASSVTPPAPAREPGHSPQIISFFLALTS TALLFLLFFLTIRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRF PEEEEGGCEL	4-1BB costimulatory domain (Accession No. Q07011.1) Homo sapien
94	MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNLSCKYSYNLFSR EFRASLHKGLD SAVEVCVVYGNYSQQQLQVYSKTGFNCDGKLGNESVTFYLQNLVYNQTDI YFCKIEVMYPP PYLDNEKSNGTIIHVKGKHLCPSPFLPGPSKPFWVLVVVGGVLACYSLL VTVAFIIFWVR SKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS	CD28 transmembrane domain (Accession No. P10747) Homo sapien
95	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTAL FLRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGG KPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR	CD3 zeta chain (Accession No. P20963) Homo sapien
96	FIIIFWVNNKRGRKK	Synthetic peptide
97	IFWVNNKRGRKKLL	Synthetic peptide
98	FIIIFWVNNKRGRKK	Synthetic peptide
99	IFWVNNKRGRKKLL	Synthetic peptide
100	VAFIIFWVR	Synthetic peptide K28R
101	AFIIFWVRR	Synthetic peptide K28R
102	FIIIFWVARG	Synthetic peptide K28A
103	MFWVLVVVGGVLACYSLLVTVAFIIFWV	CD28 transmembrane domain (amino acids 153-179 of

		Accession No. P10747) Homo sapien
104	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLEFPGPSKPFVVLVVVGGV LACYSLLVTVAFIIFWV	CD28, including transmembrane (amino acids 114-179 of Accession No. P10747) Homo sapien
105	RVKFSRSAEPPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPGEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR	CD3 zeta Homo sapien
106	GAATCTAAGTACGGACCGCCCTGCCCCCTTGCCCT	spacer (IgG4hinge) (nt) homo sapien
107	ESKYGPPCPPCPGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCS VMHEALHNHYTQKSLSLSLGK	Hinge-CH3 spacer Homo sapien
108	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVQLHQLDNLG KEYKCKVSNKGLPSSIEKTIKAKGQPREPQVYTLPPSQEEMTKNQVSL TCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDK SRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	Hinge-CH2-CH3 spacer Homo sapien
109	RWPESPKAQASSVPTAQPPQAEGSLAKATTAPATTRNTGRGGEKKKEKE KEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWRDKATFTCFVVGSD LKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSLWNAGT SVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLASSDPPEAASWLLC EVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWAWSVLRVP APPSPQPATYTCVVSHEDSRTLLNASRSLEVSIVTDH	IgD-hinge-Fc Homo sapien
110	LEGGGEGRGSLTTCGDVEENPGPR	T2A artificial
111	MLLLVTSLLLCELPHPAFLLIPRKVCNGIGIGEFKDSLSINATNIKHFK NCTSIGDLHILPVAFRGDSFTHTPPLDPQELDILKTVKEITGFLLIQA WPENRTDLHAFENLEIIRGRKQHGQFSLAVVSLNITSLGLRSLKEISD GDVLIISGNKNLCYANTINWKKLFGTSGQKTKIISNRGENSCKATGQVCH ALCSPEGCGWPEPRDCVSCRNVSRGRECVDKCNLLEGEPPREFVENSECI QCHPECLPQAMNITCTGRGPDNCIQCAHYIDGPHCVKTCPAGVMGENNT LVWKYADAGHVCHLCHPNCTYGCTGPGLEGCTNGPKIPSIATGMVGAL LLLLVVALGIGLFM	tEGFR artificial
112	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	CD28 cytoplasmic domain (amino acids 180-220 of P10747) Homo sapien
113	RSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	CD28 cytoplasmic domain variant (LL to GG) Homo sapien
114	FWVLVVVGVLACYSLLVTVAFIIFWV <u>ARG</u> RKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCEL	CD28-4-1BB K28A variant
115	FWVLVVVGVLACYSLLVTVAFIIFWV <u>HRG</u> RKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCEL	CD28-4-1BB K28H variant
116	FWVLVVVGVLACYSLLVTVAFIIFWV <u>L</u> RGRKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCEL	CD28-4-1BB K28L variant
117	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGRKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCEL	CD28-4-1BB K28Q variant

118	FWVLVVVGVLACYSLLVTVAFIIFWV <u>S</u> RGRKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28S variant
119	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGAKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB R31A variant
120	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGHKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB R31H variant
121	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGLKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB R31L variant
122	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGNKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB R31N variant
123	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGRKKALYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB L34A variant
124	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGRKKSLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB L34S variant
125	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGAKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/R31A variant
126	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGNKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/R31N variant
127	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGSKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/R31S variant
128	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGRKKALYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/L34A variant
129	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGRKKSLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/L34S variant
130	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGNKKALYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB R31N/L34A variant
131	FWVLVVVGVLACYSLLVTVAFIIFWV <u>K</u> RGNKKSLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB R31N/L34S variant
132	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGNKKALYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/R31N/L34A variant
133	FWVLVVVGVLACYSLLVTVAFIIFWV <u>Q</u> RGNKKSLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB K28Q/R31N/L34S variant
134	FWVLVVVGVLACYSLLVTVAFIIFWVNN <u>K</u> RGRKKLLYIFKQPFMRPVQ TTQEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB with variant junction region with NN insertion
135	MFWVLVVVGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEEGGCEL	CD28-4-1BB
136	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPFPGPSKPFWVLVVVGVL ACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRF PEEEEEGGCEL	CD28-4-1BB
137	SLLVTVAFIIFWV <u>K</u> RGRKKLLYIFKQ	CD28-4-1BB junction region
138	SLLVTVAFIIFWV <u>A</u> RGRKKLLYIFKQ	CD28-4-1BB junction region K14A variant
139	SLLVTVAFIIFWV <u>H</u> RGRKKLLYIFKQ	CD28-4-1BB junction region K14H variant

140	SLLVTVAFIIFWV <u>L</u> RGRRKKLLYIFKQ	CD28-4-1BB junction region K14L variant
141	SLLVTVAFIIFWV <u>Q</u> RGRRKKLLYIFKQ	CD28-4-1BB junction region K14Q variant
142	SLLVTVAFIIFWV <u>S</u> RGRRKKLLYIFKQ	CD28-4-1BB junction region K14S variant
143	SLLVTVAFIIFWV <u>K</u> RGAKKLLYIFKQ	CD28-4-1BB junction region R17A variant
144	SLLVTVAFIIFWV <u>K</u> RGHKKLLYIFKQ	CD28-4-1BB junction region R17H variant
145	SLLVTVAFIIFWV <u>K</u> RG LKKLLYIFKQ	CD28-4-1BB junction region R17L variant
146	SLLVTVAFIIFWV <u>K</u> RG NKKLLYIFKQ	CD28-4-1BB junction region R17N variant
147	SLLVTVAFIIFWV <u>K</u> RGRRKKALYIFKQ	CD28-4-1BB junction region L20A variant
148	SLLVTVAFIIFWV <u>K</u> RGRRKKSLYIFKQ	CD28-4-1BB junction region L20S variant
149	SLLVTVAFIIFWV <u>Q</u> RGAKKLLYIFKQ	CD28-4-1BB junction region K14Q/R17A variant
150	SLLVTVAFIIFWV <u>Q</u> RG NKKLLYIFKQ	CD28-4-1BB junction region K14Q/R17N variant
151	SLLVTVAFIIFWV <u>Q</u> RGSKKLLYIFKQ	CD28-4-1BB junction region K14Q/R17S variant
152	SLLVTVAFIIFWV <u>Q</u> RGRRKKALYIFKQ	CD28-4-1BB junction region K14Q/L20A variant
153	SLLVTVAFIIFWV <u>Q</u> RGRRKKSLYIFKQ	CD28-4-1BB junction region K14Q/L20S variant
154	SLLVTVAFIIFWV <u>K</u> RG NKKALYIFKQ	CD28-4-1BB junction region R17N/L20A variant
155	SLLVTVAFIIFWV <u>K</u> RG NKKSLYIFKQ	CD28-4-1BB junction region R17N/L20S variant
156	SLLVTVAFIIFWV <u>Q</u> RG NKKALYIFKQ	CD28-4-1BB junction region K14Q/R17N/L20A variant
157	SLLVTVAFIIFWV <u>Q</u> RG NKKSLYIFKQ	CD28-4-1BB junction region K14Q/R17N/L20S variant
158	G A A T C T A A G T A C G G A C C G C C C T G C C	spacer (IgG4hinge)

	C C C C T T G C C C T	(nucleotide) homo sapien
159	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR	CD3 Zeta
160	CYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQT	peptide
161	CYSLLVTVAFIIFWV	Synthetic peptide
162	LLVTVAFIIFWVKRG	Synthetic peptide
163	TVAFIIFWVKRGRKK	Synthetic peptide
164	FIIFWVKRGRKKLLY	Synthetic peptide
165	FWVKRGRKKLLYIFK	Synthetic peptide
166	KRGRKKLLYIFKQPF	Synthetic peptide
167	RKKLLYIFKQPFMRP	Synthetic peptide
168	LLYIFKQPFMRPVQT	Synthetic peptide
169	TVAFIIFWVKRGHKK	Synthetic peptide R31H
170	FIIFWVKRGHKKLLY	Synthetic peptide R31H
171	FWVKRGHKKLLYIFK	Synthetic peptide R31H
172	KRGHKKLLYIFKQPF	Synthetic peptide R31H
173	HKKLLYIFKQPFMRP	Synthetic peptide R31H
174	TVAFIIFWVKRGNKK	Synthetic peptide R31N
175	FIIFWVKRGNKKLLY	Synthetic peptide R31N
176	FWVKRGNKKLLYIFK	Synthetic peptide R31N
177	KRGNKKLLYIFKQPF	Synthetic peptide R31N
178	NKKLLYIFKQPFMRP	Synthetic peptide R31N
179	FIIFWVKRGRKKALY	Synthetic peptide L34A
180	FWVKRGRKKALYIFK	Synthetic peptide L34A
181	KRGRKKALYIFKQPF	Synthetic peptide L34A
182	RKKALYIFKQPFMRP	Synthetic peptide L34A
183	FWVLVVVGVLACYSLLVTVAFIIFWVKRGSKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCEL	CD28-4-1BB R31S variant
184	SLLVTVAFIIFWVKRGSKKLLYIFKQ	CD28-4-1BB junction region R31S variant

CLAIMS

WHAT IS CLAIMED:

1. A variant chimeric receptor, comprising a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein:

the reference chimeric receptor comprises a first domain and a second domain, joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor comprises up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and

a peptide fragment having the sequence of an 8-15 amino acid portion of the modified junction region has a binding affinity for a human leukocyte antigen (HLA) molecule that is lower than the binding affinity, for the same HLA molecule, of a peptide fragment having the sequence of the corresponding portion of the junction region of the reference chimeric receptor.

2. The variant chimeric receptor of claim 1, wherein the peptide fragment of the corresponding portion of the junction region of the reference chimeric receptor has a binding affinity of less than 1000 nM, less than 500 nM or less than 50 nM.

3. A variant chimeric receptor, comprising a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein:

the reference chimeric receptor comprises a first domain and a second domain, joined in contiguous sequence at a junction, wherein the junction region of the reference chimeric receptor comprises up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and

the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the modified junction region for a human HLA molecule is lower than the average of the binding affinities of all 8-15 amino acid fragments, or of all 8, 9, 10, 11, 12, 13, 14, or 15 amino acid fragments, within the junction region of the reference chimeric receptor.

4. The variant chimeric receptor of any of claims 1-3, wherein the binding affinity or average of binding affinities is more than 2-fold, more than 5-fold, more than 10-fold, more than 25-fold, more than 50-fold or more than 100-fold lower.

5. A variant chimeric receptor, comprising a modified junction region having one or more amino acid sequence modifications compared to a junction region of a reference chimeric receptor, wherein:

the reference chimeric receptor comprises a first domain directly linked to a second domain joined in contiguous sequence at a junction, wherein;

the junction region of the reference chimeric receptor comprises up to 15 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction; and

the number of peptide fragments having the sequence of an 8-15 amino acid portion of the modified junction region that has a binding affinity for a human leukocyte antigen (HLA) of less than 1000 nM is reduced compared to the number of peptide fragments having the sequence of an 8-15 amino acid portion of the junction region of the reference chimeric receptor that has the same affinity for binding the same HLA.

6. The variant chimeric receptor of claim 5, wherein:

the number of peptide fragments within the modified junction region that exhibits a binding affinity for an HLA of less than 500 nM or less than 50 nM is reduced; or

the binding affinity of less than 1000 nM is a binding affinity of less than 500 nM or less than 50 nm.

7. The variant chimeric receptor of any of claims 1-6, wherein the binding affinity is an IC₅₀ and the comparison of binding of peptide fragments of the modified junction regions and the peptide fragments of the junction region of the reference chimeric receptor is with reference to the same standard peptide.

8. The variant chimeric receptor of any of claims 1-7, wherein:

the first domain and/or second domain comprises a domain of a natural human protein; and/or

the first domain and/or second domain comprises an extracellular binding domain, a hinge domain, a transmembrane domain, or an intracellular signaling domain, which intracellular signaling domain is, optionally, a costimulatory signaling domain or an activating cytoplasmic signaling domain.

9. The variant chimeric receptor of any of claims 1-8, wherein the first domain and second domain are not present in the same molecule *in vivo* in a human subject.

10. The variant chimeric receptor of any of claims 1-9, wherein the first domain and second domain are, respectively, an extracellular ligand binding domain and a hinge domain, a hinge domain and a transmembrane domain, a transmembrane domain and an intracellular costimulatory signaling domain, and an intracellular costimulatory signaling domain and an activating cytoplasmic signaling domain, which can include functional portions of such domains.

11. The variant chimeric receptor of any of claims 1-10, wherein the first domain is a transmembrane domain or a functional portion thereof and the second domain is a costimulatory signaling domain or a functional portion thereof.

12. The variant chimeric receptor of claim 11, wherein the transmembrane domain is a CD28 transmembrane domain or a functional portion or variant thereof and the costimulatory signaling domain is a 4-1BB signaling domain or a functional portion or variant thereof.

13. The variant chimeric receptor of any of claims 1-12, wherein the junction region of the reference chimeric receptor comprises up to 13 contiguous amino acids directly C-terminal of the junction and/or up to 15 contiguous amino acids directly N-terminal of the junction.

14. The variant chimeric receptor of any of claims 1-13, wherein the peptide fragment(s) comprises a sequence of amino acids between or between about 8 and 15 amino acids in length, or comprises a sequence of amino acids that is at least or at least about or is or is about 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in length.

15. The variant chimeric receptor of any of claims 1-14, wherein the variant chimeric receptor comprises:

a domain of at least 95% sequence identity to the first domain and/or a domain of at least 95% sequence identity to the second domain;

a domain identical in sequence to the first domain and a domain of at least 95% sequence identity to the second domain; or

a domain of at least 95% sequence identity to the first domain and a domain identical in sequence to the second domain,

wherein at least one or both of the domains present in the variant chimeric receptor is modified compared to the first domain and/or second domain of the reference chimeric receptor in the portion comprising the modified junction region.

16. The variant chimeric receptor of any of claims 1-15, wherein:

the variant chimeric receptor comprises at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the reference chimeric receptor; and/or

the variant chimeric receptor comprises up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid modifications compared to the reference chimeric receptor.

17. The variant chimeric receptor of any of claims 12-16, wherein

the CD28 transmembrane domain comprises the sequence of amino acids set forth in SEQ ID NO:2, 103 or 104 or a functional portion or variant thereof comprising a sequence that exhibits at least 95% sequence identity to SEQ ID NO:2, 103 or 104; and

the 4-1BB costimulatory signaling domain comprises the sequence of amino acids set forth in SEQ ID NO:3 or a functional portion or variant thereof comprising a sequence that exhibits at least 95% sequence identity to SEQ ID NO:3.

18. The variant chimeric receptor of any of claims 12-17, wherein the first domain and second domain together comprise the sequence of amino acids set forth in SEQ ID NO:5 or a functional portion or variant thereof comprising a sequence of amino acids that exhibits at least

85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO:5.

19. The variant chimeric receptor of any of claims 12-18, wherein the first domain and second domain together comprise the sequence of amino acids set forth in SEQ ID NO:5.

20. The variant chimeric receptor of any of claims 12-19, wherein the one or more modifications are within a portion between residue 13 and 42 or between amino acid residue 15 and 40, with reference to numbering set forth in SEQ ID NO:5.

21. The variant chimeric receptor of any of claims 1-20, wherein the one or more modifications comprises an amino acid insertion, replacement or deletion and/or wherein each of the one or more modifications individually comprises an amino acid insertion, replacement, or deletion.

22. The variant chimeric receptor of any of claims 1-21, wherein the first and/or second domain comprises a transmembrane domain, wherein

the one or more modifications is not or does not comprise a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, which optionally is the CD28 transmembrane domain; or

the one or more modifications comprises a modification of a hydrophobic amino acid residue within the transmembrane domain, which optionally is the CD28 transmembrane domain, wherein the modification is or comprises a substitution of the hydrophobic amino acid with another different hydrophobic amino acid residue; or

the one or more modifications is not or does not comprise a modification at or of a hydrophobic amino acid residue or within a hydrophobic portion in the transmembrane domain, which optionally is the CD28 transmembrane domain, other than a substitution with another hydrophobic amino acid residue.

23. The variant chimeric receptor of any of claims 12-22, wherein the one or more modifications comprise a modification at an amino acid residue corresponding to an amino acid residue between residues 28 and 42 with reference to numbering set forth in SEQ ID NO:5.

24. The variant chimeric receptor of any of claims 1-23, wherein the amino acid modification is an insertion and the variant chimeric receptor comprises an insertion between amino acid residues adjacent to the junction between the domains, which optionally corresponds to amino acid residues 27 and 28 with reference to numbering set forth SEQ ID NO:5.

25. The variant chimeric receptor of claim 24, comprising insertion of 1, 2, 3, 4 or 5 amino acid residues.

26. The variant chimeric receptor of claim 24 or claim 25, wherein the insertion is of any amino acid residue, which, optionally, is asparagine (N).

27. The variant chimeric receptor of any of claims 12-26, wherein the one or more modifications comprises an amino acid replacement(s) and the amino acid replacement(s) are at one or more residues corresponding to a residue selected from 28, 31 or 34 with reference to numbering set forth in SEQ ID NO:5.

28. The variant chimeric receptor of claim 27, wherein the amino acid replacement(s) is to any other amino acid residue, which, optionally, is selected from among leucine (L), asparagine (N), glutamine (Q), alanine (A), serine (S) or histidine (H).

29. The variant chimeric receptor of claim 27 or claim 28, wherein the amino acid replacement(s) corresponds to or is a replacement selected from among K28A, K28H, K28L, K28Q, K28S, R31A, R31H, R31L, R31N, R31S, L34A and L34S, with reference to numbering set forth in SEQ ID NO:5.

30. The variant chimeric receptor of any of claims 27-29, wherein the amino acid replacement does not comprise the single amino acid replacement corresponding to L34A or L34S, with reference to numbering set forth in SEQ ID NO:5.

31. The variant chimeric receptor of any of claims 27-30, wherein the amino acid replacements are or correspond to amino acid replacements selected from among K28Q/R31A, K28Q/R31N, K28Q/R31S, K28Q/L34A, K28Q/L34S, R31N/L34A, R31N/L34S, K28Q/R31N/L34A, K28Q/R31N/L34S.

32. The variant chimeric receptor of any of claims 1-31, wherein:
the variant chimeric receptor comprises a modified junction region comprising less than 100% sequence identity to SEQ ID NO:137 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% to SEQ ID NO:137 and comprises the modifications; and/or
the variant chimeric receptor comprises a sequence comprising less than 100% sequence identity to SEQ ID NO:5 but greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95% or 96% to SEQ ID NO:5 and comprises the modifications.

33. The variant chimeric receptor of any of claims 1-32, wherein the variant chimeric receptor comprises a modified junction region selected from among:

- i) the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184;
- ii) a functional variant thereof comprising a sequence of amino acids that exhibits at least 95% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 138-157 and 184 and that comprise the modification(s); or
- iii) a functional portion of i) or ii) and comprises the modification(s).

34. The variant chimeric receptor of any of claims 1-33, wherein the variant chimeric receptor comprises:

- i) the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183;
- ii) a functional variant thereof comprising a sequence of amino acids that exhibits at least 95% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS: 114-134 and 183 and that comprise the modification(s);
- ii) or is a functional portion of i) or ii) and comprises the modification(s).

35. The variant chimeric receptor of any of claims 1-34, wherein the modified junction region comprises no more than 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications compared to the junction region of the reference chimeric receptor.

36. The variant chimeric receptor of any of claims 1-35, wherein the first domain or second domain is a transmembrane domain and the corresponding domain in the variant chimeric receptor comprises a substantially hydrophobic hydropathy profile and/or comprises a grand average of hydropathy (GRAVY) value of greater than 0, 0.25, 0.5, 0.75, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2 or greater.

37. The variant chimeric receptor of any of claims 1-36, wherein first domain or second domain comprises an intracellular signaling domain and the corresponding domain in the variant chimeric receptor is capable of inducing the activation or cellular localization of a TRAF and/or is capable of inducing TRAF-mediated signaling.

38. The variant chimeric receptor of claim 37, wherein the intracellular signaling domain is a 4-1BB co-stimulatory signaling domain and/or the TRAF is selected from among TRAF1, TRAF2 or TRAF3.

39. The variant chimeric receptor of any of claims 12-38, wherein the variant chimeric receptor comprises amino acids TTQE at positions corresponding to 49-52 and/or amino acids PEEE at positions corresponding to residues 60-63, each with reference to numbering set forth in SEQ ID NO:5.

40. The variant chimeric receptor of any of claims 1-39, wherein the HLA is an HLA class I and/or an HLA class II.

41. The variant chimeric receptor of any of claims 1-40, wherein the HLA class I is selected from an HLA allele set forth in Table 1A and/or the HLA class II allele is selected from an HLA allele set forth in Table 1B.

42. The variant chimeric receptor of any of claims 1-41, wherein the HLA class I allele is selected from among HLA-A*02:01, HLA-A*03:01, HLA-A*11:01 and HLA-B*08:01.

43. The variant chimeric receptor of any of claims 3-42, wherein the HLA comprises a plurality of HLA molecules and average binding affinities to one or more of the plurality of HLA molecules is lower and/or the number of peptide fragments that individually bind to one or more of the plurality of HLA molecules is reduced.

44. The variant chimeric receptor of claim 43, wherein the plurality of HLA molecules is selected from:

a plurality of HLA class I molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class I molecules in the worldwide population or in the Caucasian population;

a plurality of HLA class II molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class II molecules in the worldwide population or in the Caucasian population; or

a plurality of HLA class I molecules and HLA class II molecules that represent greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 95% or greater than 99% of the HLA class I molecules and HLA class II molecules in the worldwide population or in the Caucasian population;

45. The variant chimeric receptor of any of claims 1-44, wherein the binding affinity is as determined *in vitro*.

46. The variant chimeric receptor of any of claims 1-45 that exhibits reduced immunogenicity compared to the reference chimeric receptor upon administration to a human subject, optionally wherein the subject has received administration of the reference chimeric receptor.

47. The variant chimeric receptor of claim 46, wherein the reduced immunogenicity comprises a reduced CD4+ T cell immune response and/or a reduced CD8+ T cell immune response.

48. The variant chimeric receptor of any of claims 11-47, wherein:
the reference chimeric receptor further comprises an extracellular ligand-binding domain; and/or
the variant chimeric receptor further comprises an extracellular ligand-binding domain.
49. The variant chimeric receptor of claim 48 that is a chimeric antigen receptor (CAR), wherein the ligand-binding domain is an antigen-binding domain.
50. The variant chimeric receptor of claim 49, wherein the antigen-binding domain is an antibody or an antibody fragment.
51. The variant chimeric receptor of claim 50, wherein the antigen-binding domain is an antibody fragment that is a single chain fragment.
52. The variant chimeric receptor of claim 50 or claim 51, wherein the fragment comprises antibody variable regions joined by a flexible immunoglobulin linker.
53. The variant chimeric receptor of any of claims 50-52, wherein the antibody fragment comprises an scFv.
54. The variant chimeric receptor of any of claims 48-52, wherein the ligand-binding domain specifically binds an antigen that is associated with a disease or disorder.
55. The variant chimeric receptor of claim 54, wherein:
the disease or disorder is an infectious disease or condition, an autoimmune disease, an inflammatory disease or a tumor or a cancer;
the ligand-binding domain specifically binds to a tumor antigen; and/or
the ligand-binding domain specifically binds to an antigen selected from the group consisting of ROR1, Her2, LI-CAM, CD19, CD20, CD22, mesothelin, CEA, hepatitis B surface antigen, anti-folate receptor, CD23, CD24, CD30, CD33, CD38, CD44, EGFR, EGP-2, EGP-4, EPHA2, ErbB2, ErbB3, ErbB4, FBP, fetal acetylcholine e receptor, GD2, GD3, HMW-MAA, IL-22R-alpha, IL-13R-alpha2, kdr, kappa light chain, Lewis Y, L1-cell adhesion molecule,

MAGE-A1, mesothelin, MUC1, MUC16, PSCA, NKG2D Ligands, NY-ESO-1, MART-1, gp100, oncofetal antigen, TAG72, VEGF-R2, carcinoembryonic antigen (CEA), prostate specific antigen, PSMA, estrogen receptor, progesterone receptor, ephrinB2, CD123, CS-1, c-Met, GD-2, MAGE A3, CE7, Wilms Tumor 1 (WT-1) and cyclin A1 (CCNA1).

56. The variant chimeric receptor of any of claims 11-55, wherein:
the reference chimeric receptor further comprises an activating cytoplasmic signaling domain; and/or
the variant chimeric receptor further comprises an activating cytoplasmic domain.

57. The variant chimeric receptor of claim 56, wherein the activating cytoplasmic domain comprises a T cell receptor (TCR) component and/or comprise an immunoreceptor tyrosine-based activation motif (ITAM).

58. The variant chimeric receptor of claim 56 or claim 57, wherein the activating cytoplasmic signaling domain is or comprises a cytoplasmic signaling domain of a zeta chain of a CD3-zeta (CD3 ζ) chain or a functional variant or signaling portion thereof.

59. The variant chimeric receptor of any of claims 11-58, wherein:
the reference chimeric receptor comprises from its N to C terminus in order: an extracellular ligand-binding domain, the first domain that is a transmembrane domain, the second domain that is an intracellular costimulatory domain and an activating cytoplasmic signaling domain; and/or

the variant chimeric receptor comprises from its N to C terminus in order: an extracellular ligand-binding domain, a transmembrane domain, a intracellular costimulatory domain and an activating cytoplasmic signaling domain, wherein the transmembrane domain and intracellular costimulatory domain are joined in contiguous sequence at a junction to form the modified junction region.

60. A nucleic acid molecule encoding the variant chimeric receptor of any of claims 1-59.

61. A vector, comprising the nucleic acid molecule of claim 60.
62. The vector of claim 61 that is a viral vector.
63. The vector of claim 61 or claim 62 that is a retroviral vector, which optionally is a lentiviral vector or a gammaretroviral vector.
64. An engineered cell, comprising the nucleic acid of claim 60 or the vector of any of claims 61-63 or expressing the chimeric receptor of any of claims 1-59.
65. The engineered cell of claim 64, which is a T cell.
66. The engineered cell of claim 64 or claim 65 that is a CD4⁺ T cell and/or a CD8⁺ T cell.
67. A composition, comprising the engineered cells of any of claims 64-66, and optionally a pharmaceutically acceptable buffer.
68. A method of treatment, comprising administering the cell of any of claims 64-66 or the composition of claim 67 to a subject having a disease or condition.
69. The method of claim 68, wherein the chimeric receptor specifically binds to a ligand or antigen associated with the disease or condition.
70. The method of claim 68 or claim 69, wherein the disease or condition is a cancer, a tumor, an autoimmune disease or disorder, or an infectious disease.
71. The method of any of claims 68-70, wherein the genetically engineered T cells in the composition exhibit increased or longer expansion and/or persistence in the subject than in a subject administered the same or about the same dosage amount of a reference cell composition expressing the reference chimeric receptor.

72. The method of claim 71, wherein the increase is at least 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, or 5-fold.

73. The method of claim 71 or claim 72, wherein the increase is observed or is present within a month, within two months, within six months or within one year of administering the cells.

FIG. 1

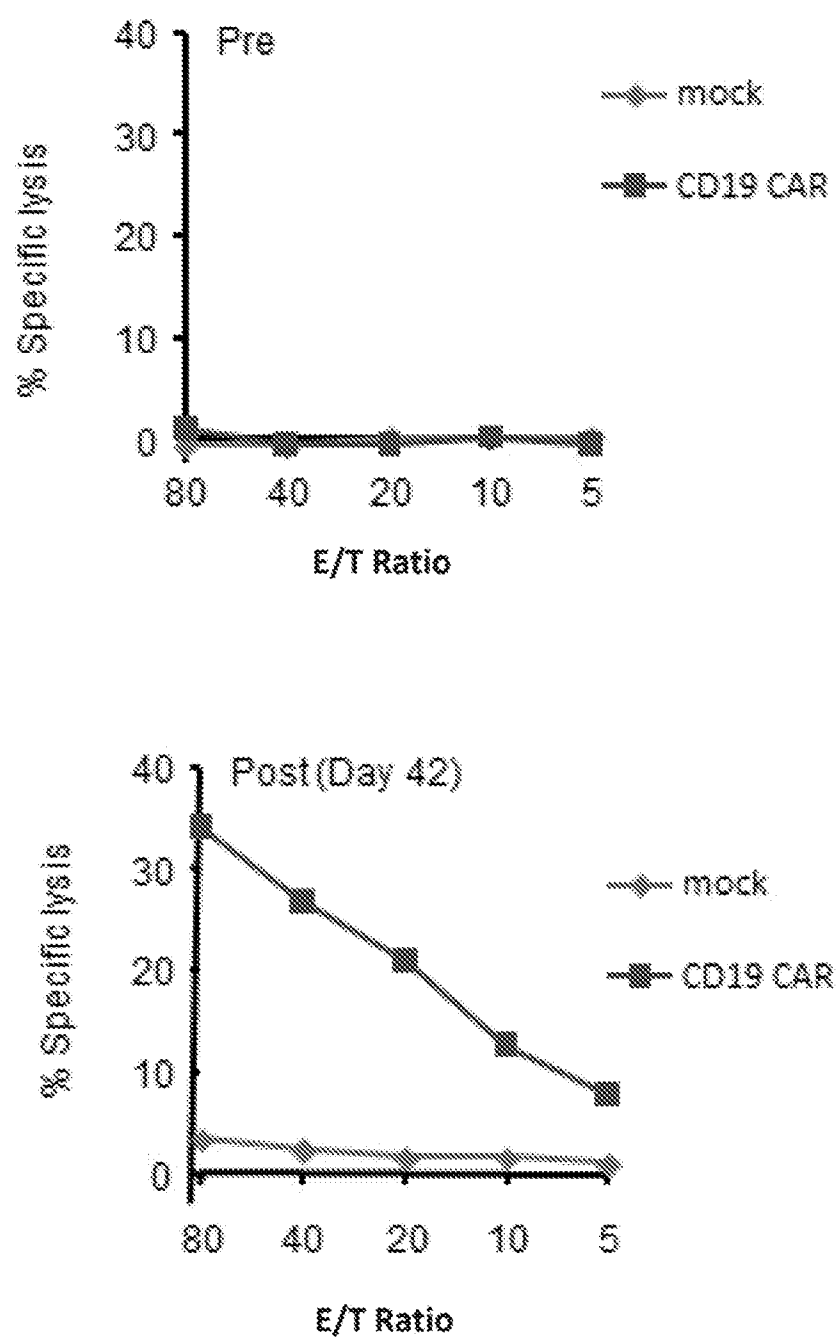


FIG. 2

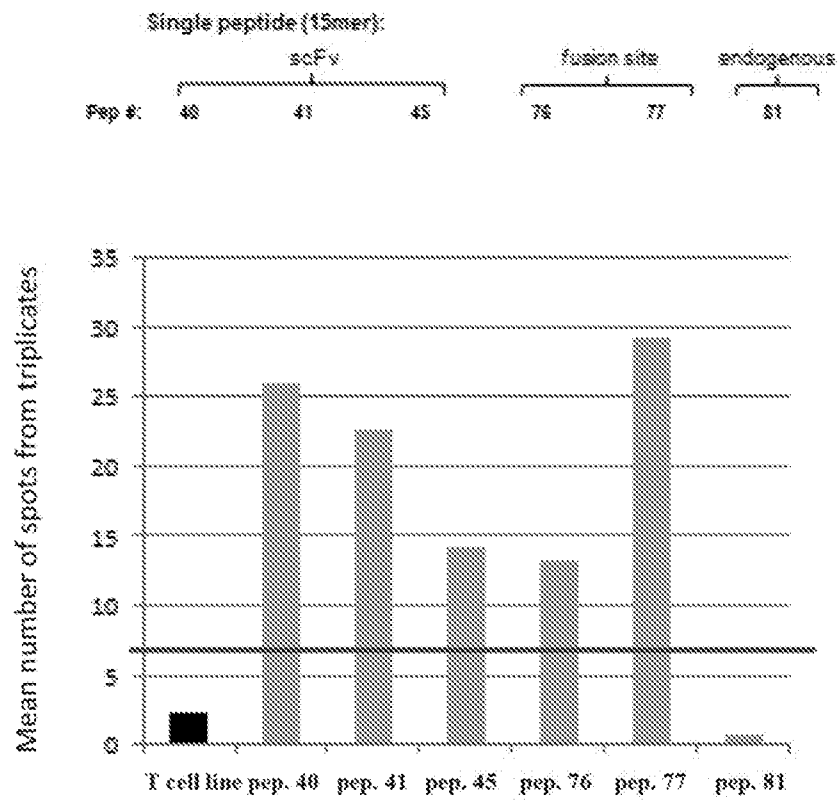


FIG. 3

Native Sequence			Variant 5		
Peptide	Region		Peptide	Region	
CYELLPTVAFITTFNNHNGKKLLSTPQGPF	Whole	143	CYELLPTVAFITTFNNHNGKKLLSTPQGPF	Whole	154
CYELLPTVAFITTFNN	Front	35	CYELLPTVAFITTFNN	Front	35
-----KNGKKLLSTPQGPF	Back	35	-----KNGKKLLSTPQGPF	Back	35
-----VKK	Fusion	73	-----VKK	Fusion	84

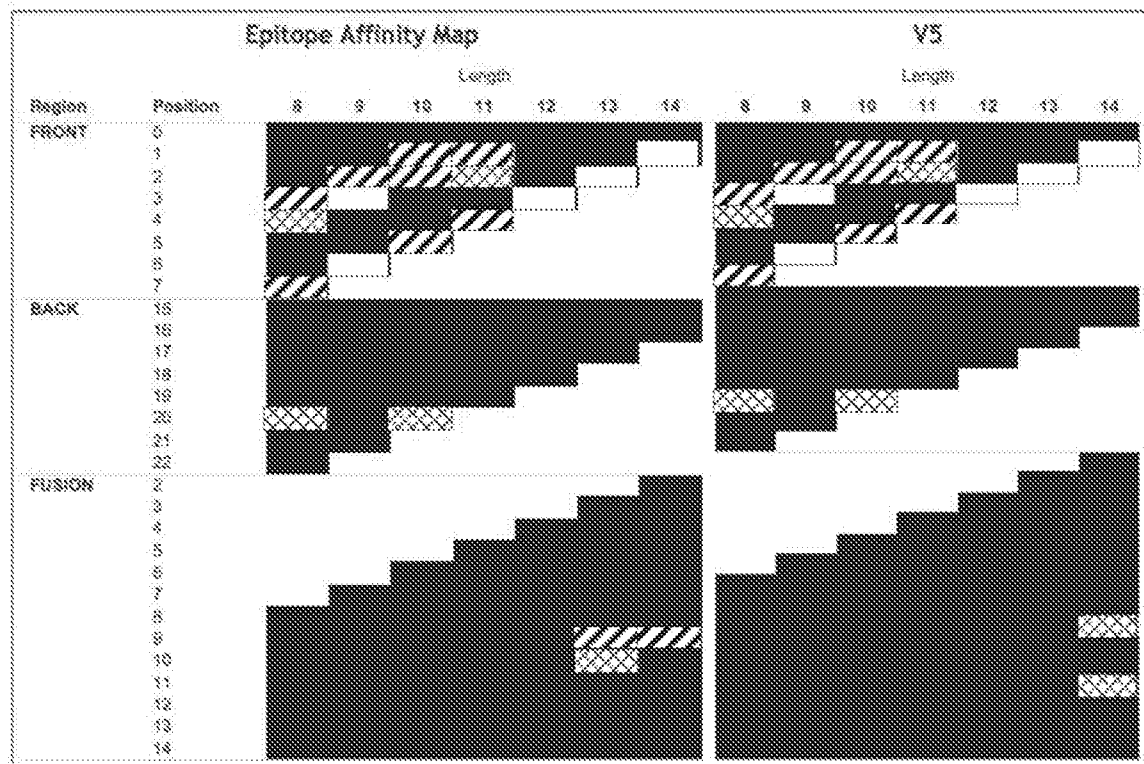


FIG. 4A

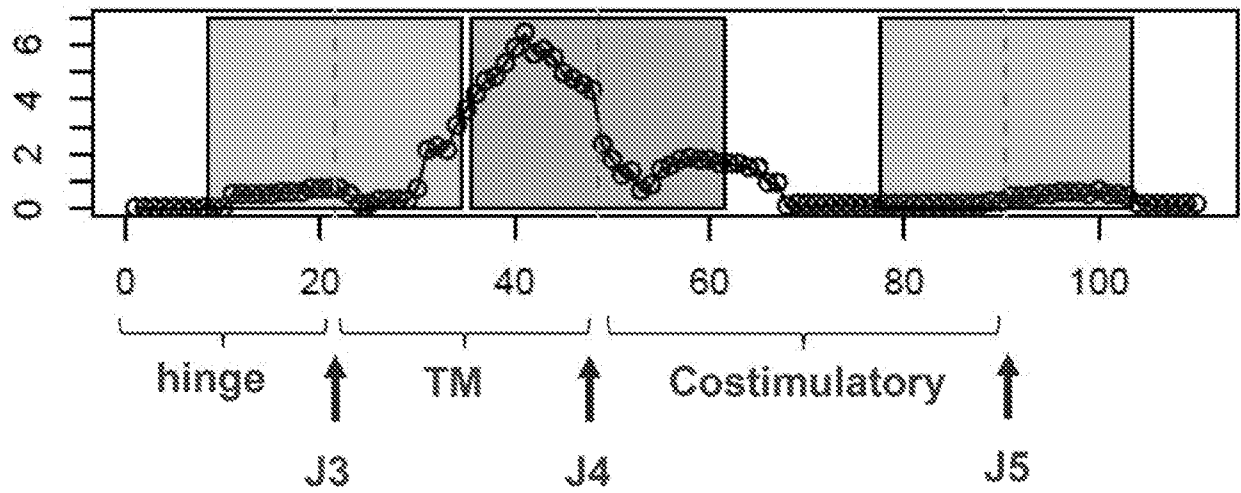


FIG. 4B

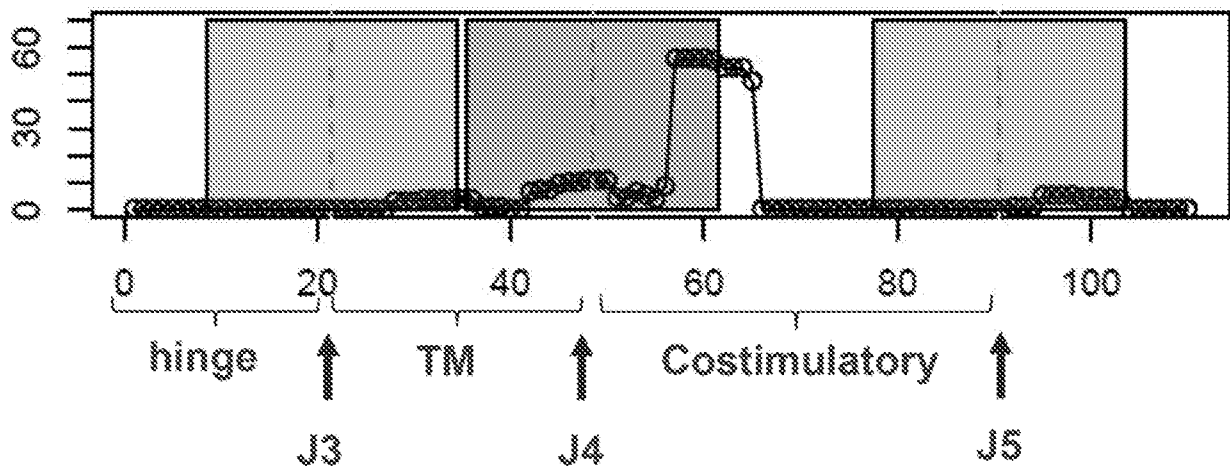


FIG. 5

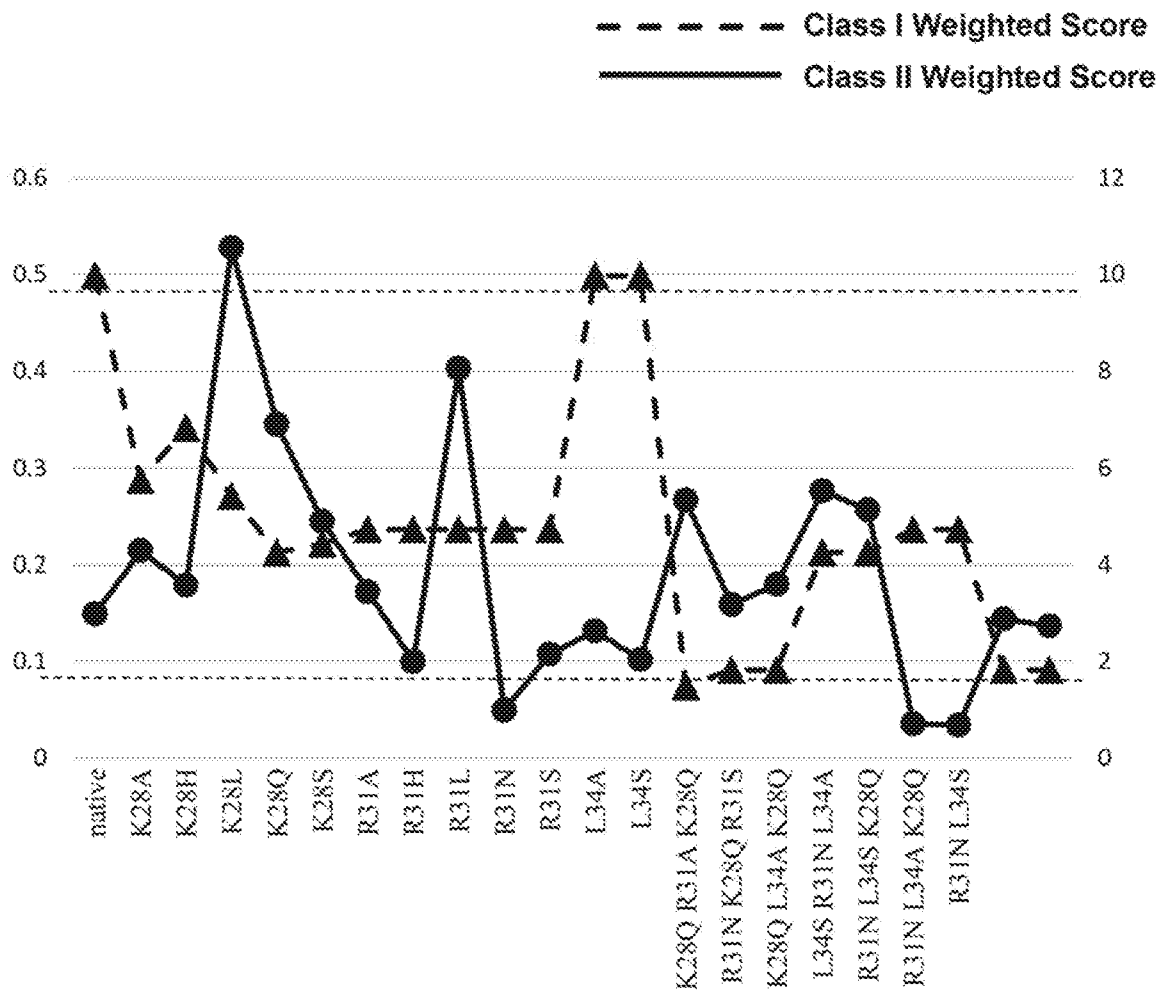
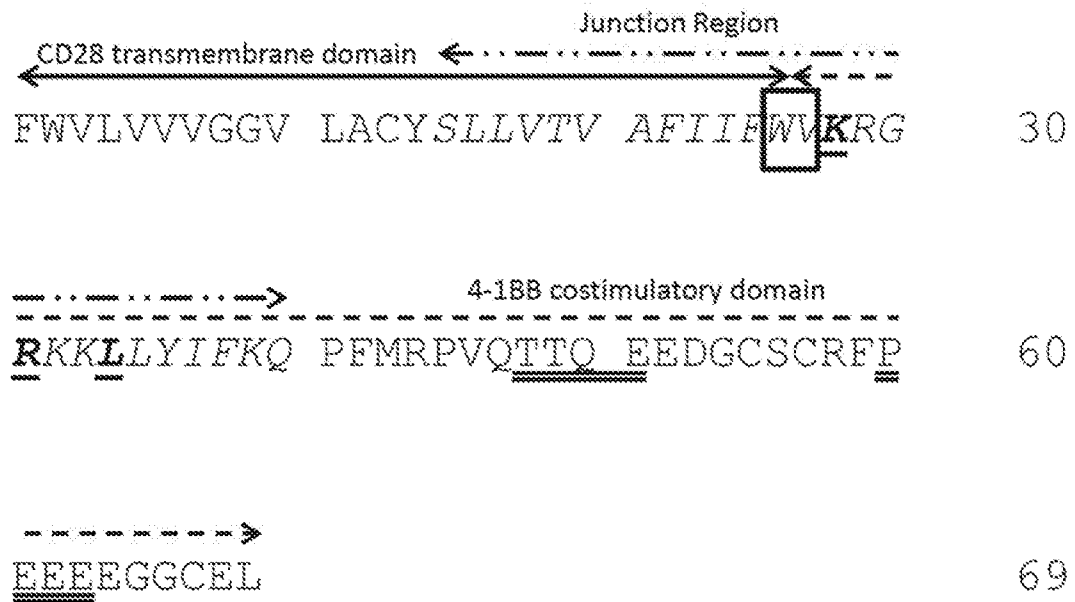


FIG. 6



SEQ ID NO: 5

FIG. 7

% Potential Impact in the Population Based on REVEAL® Score

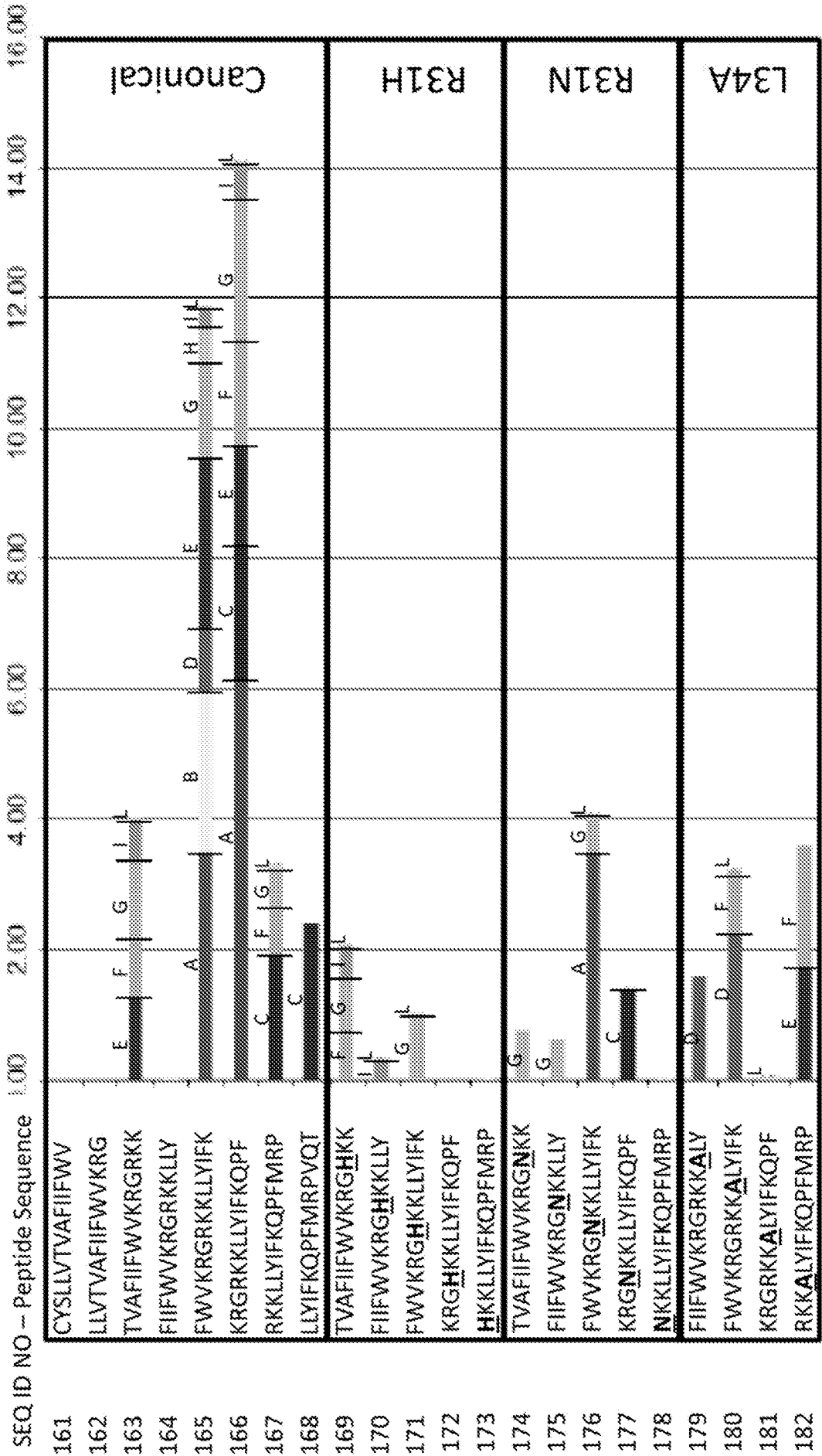
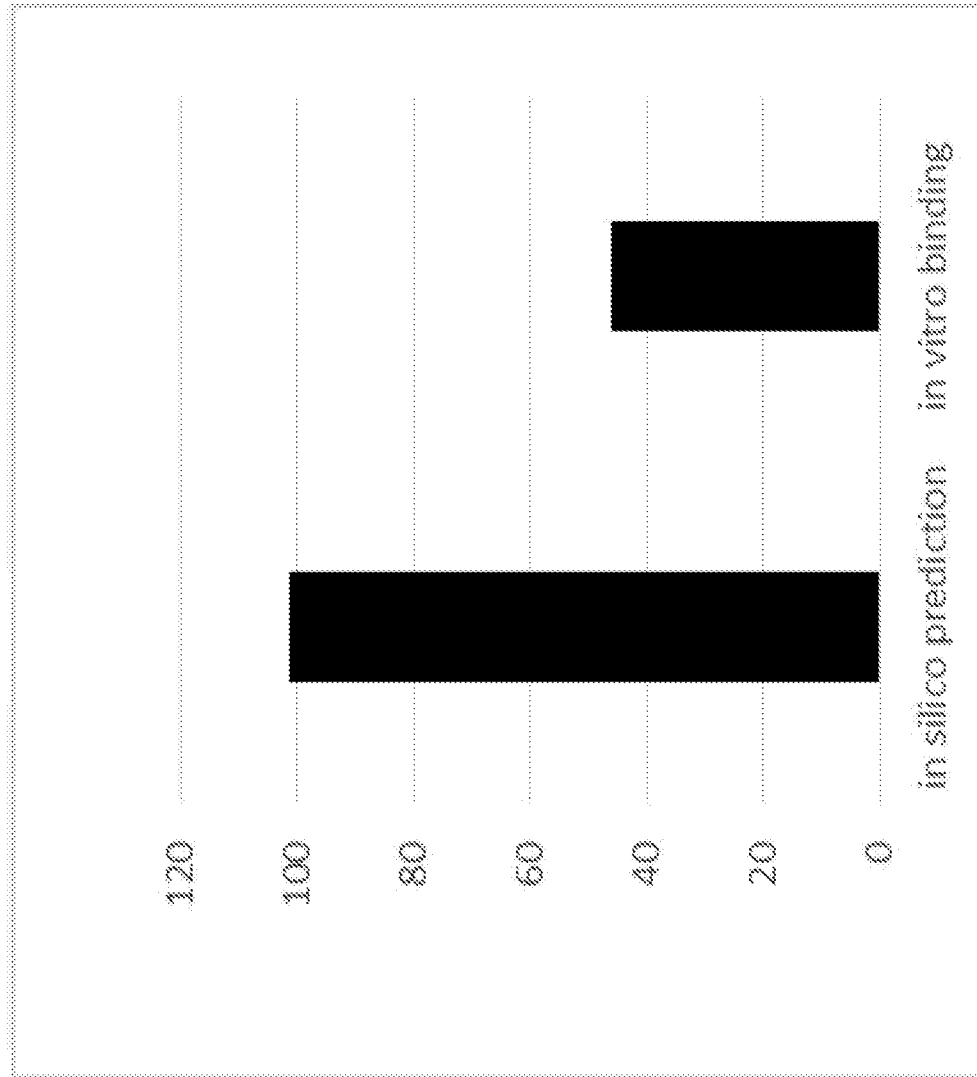
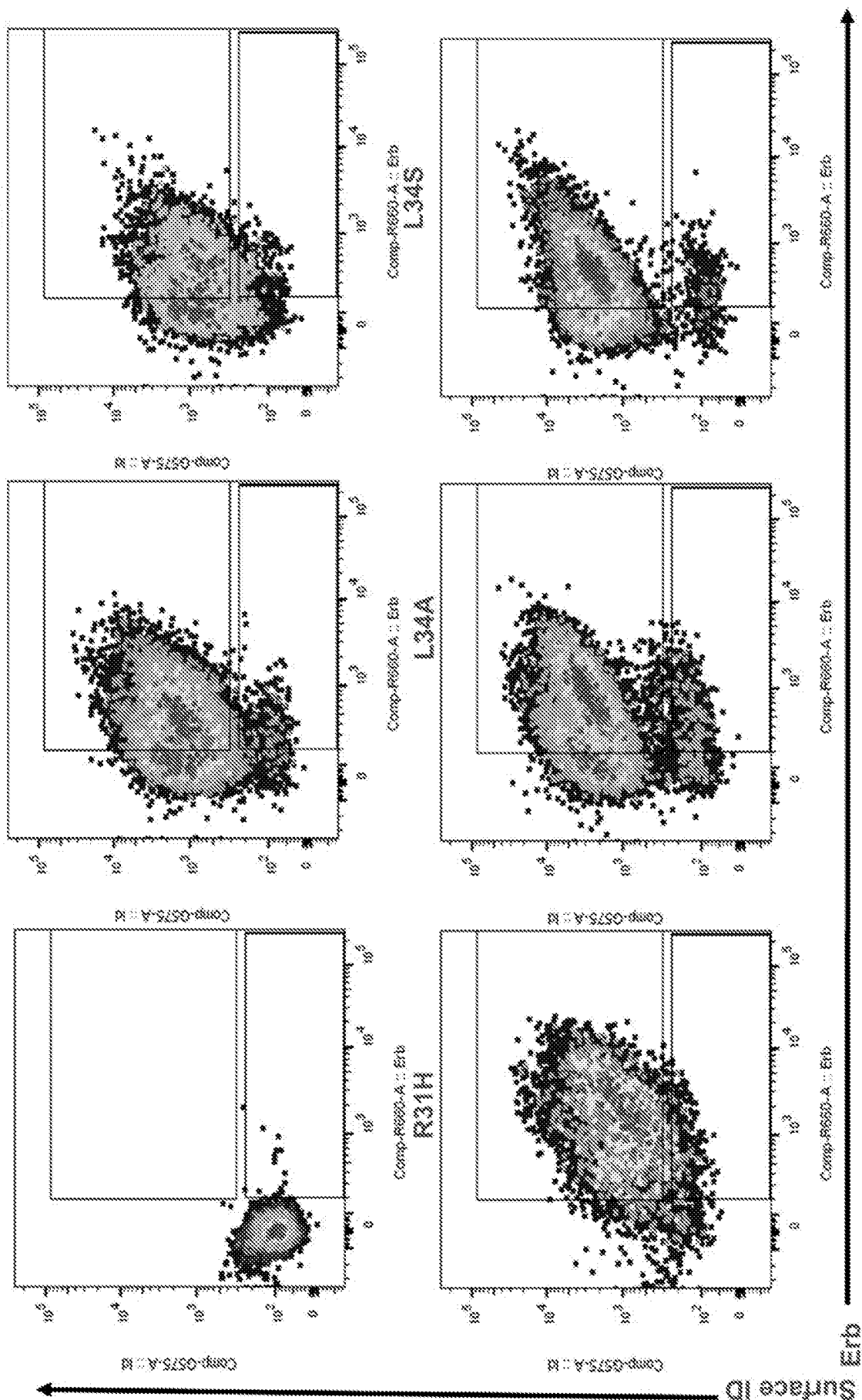
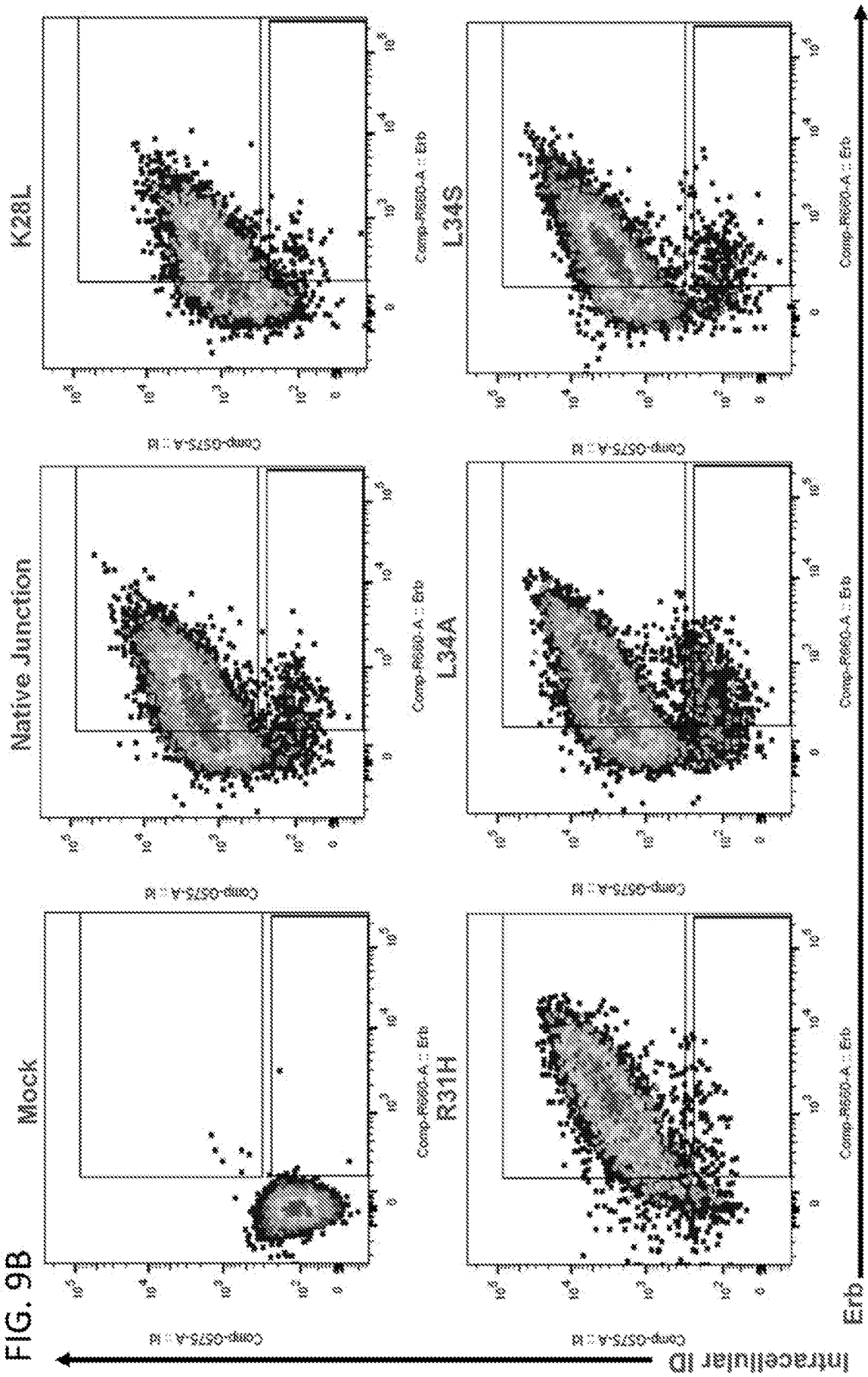


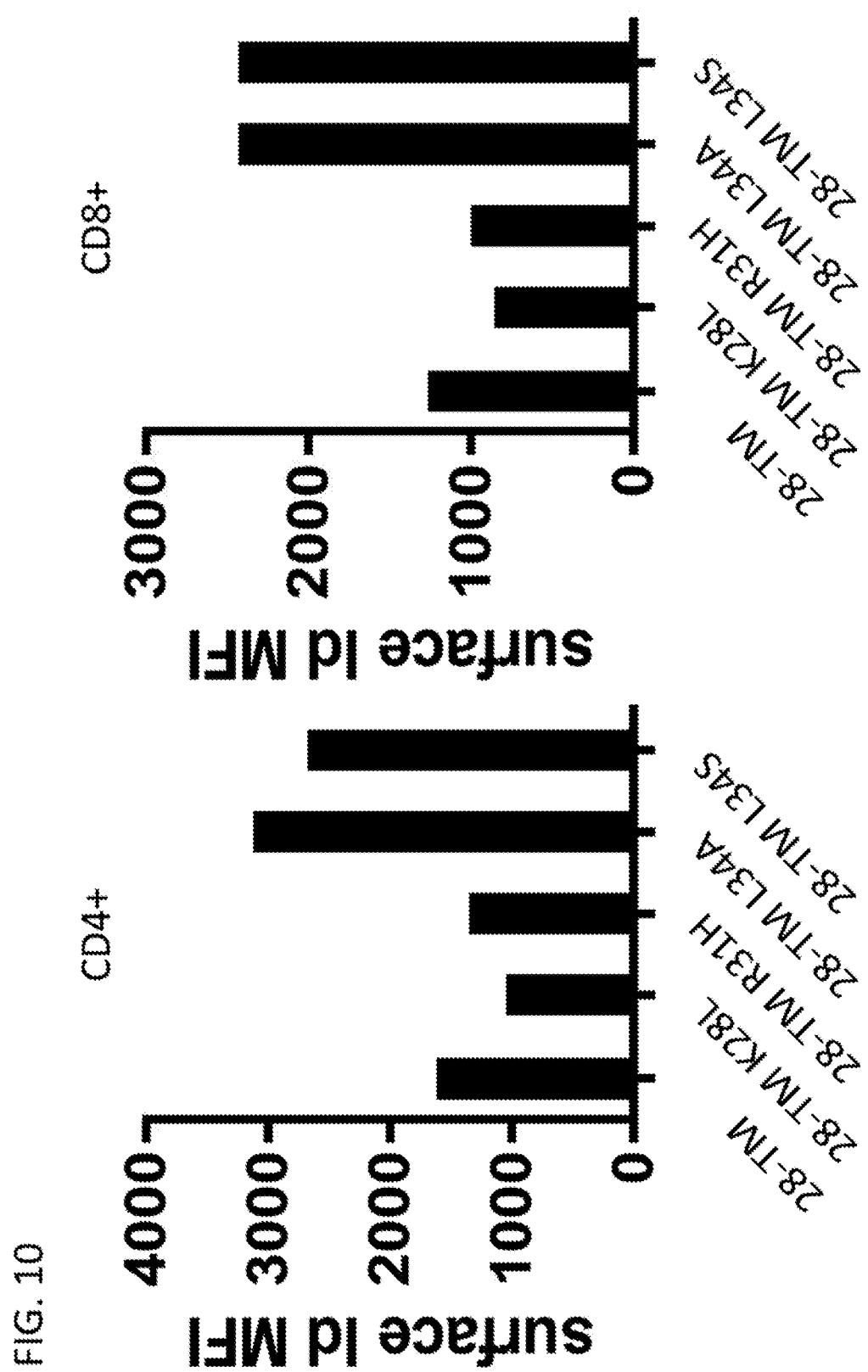
FIG. 8

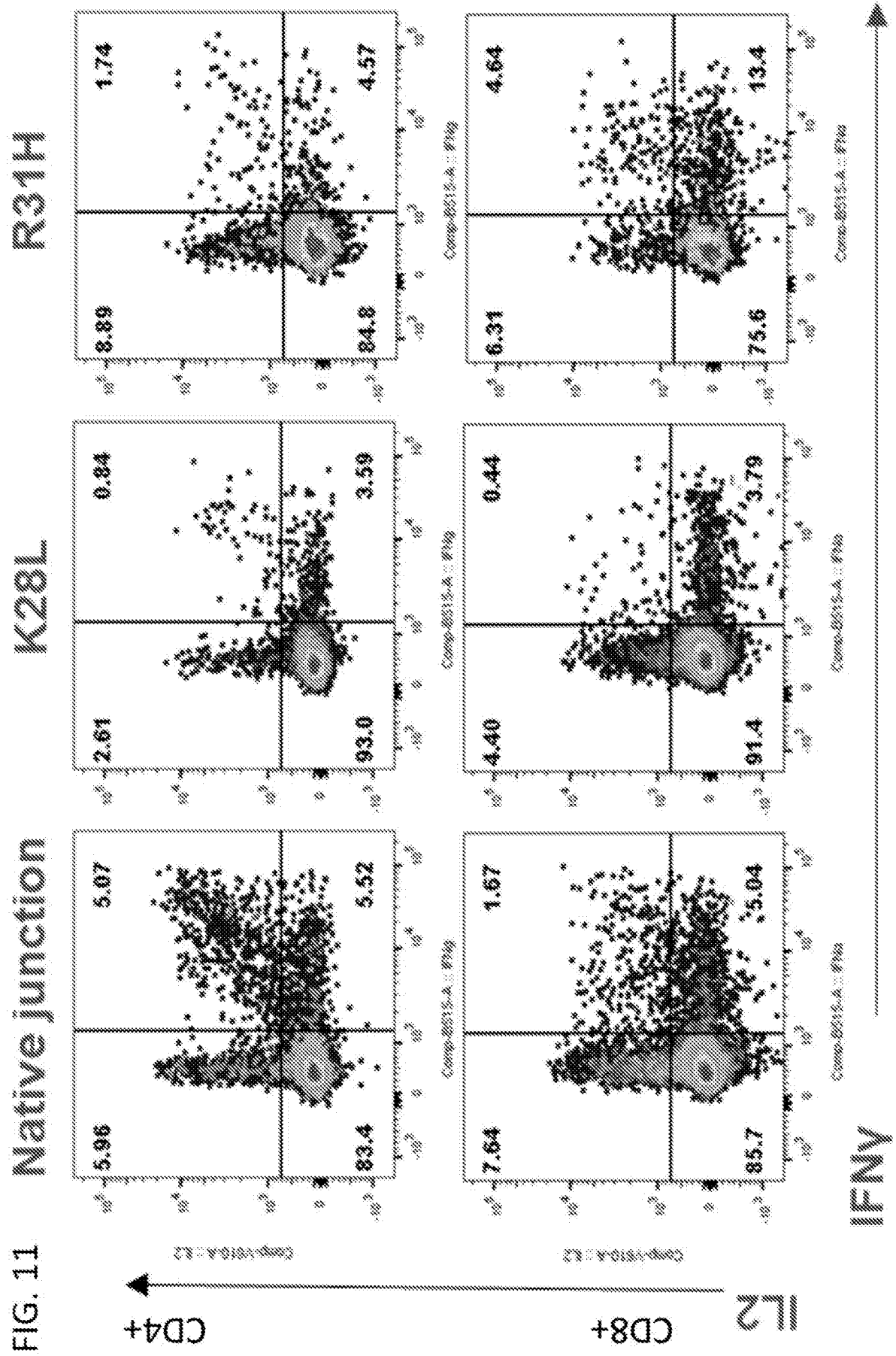


25









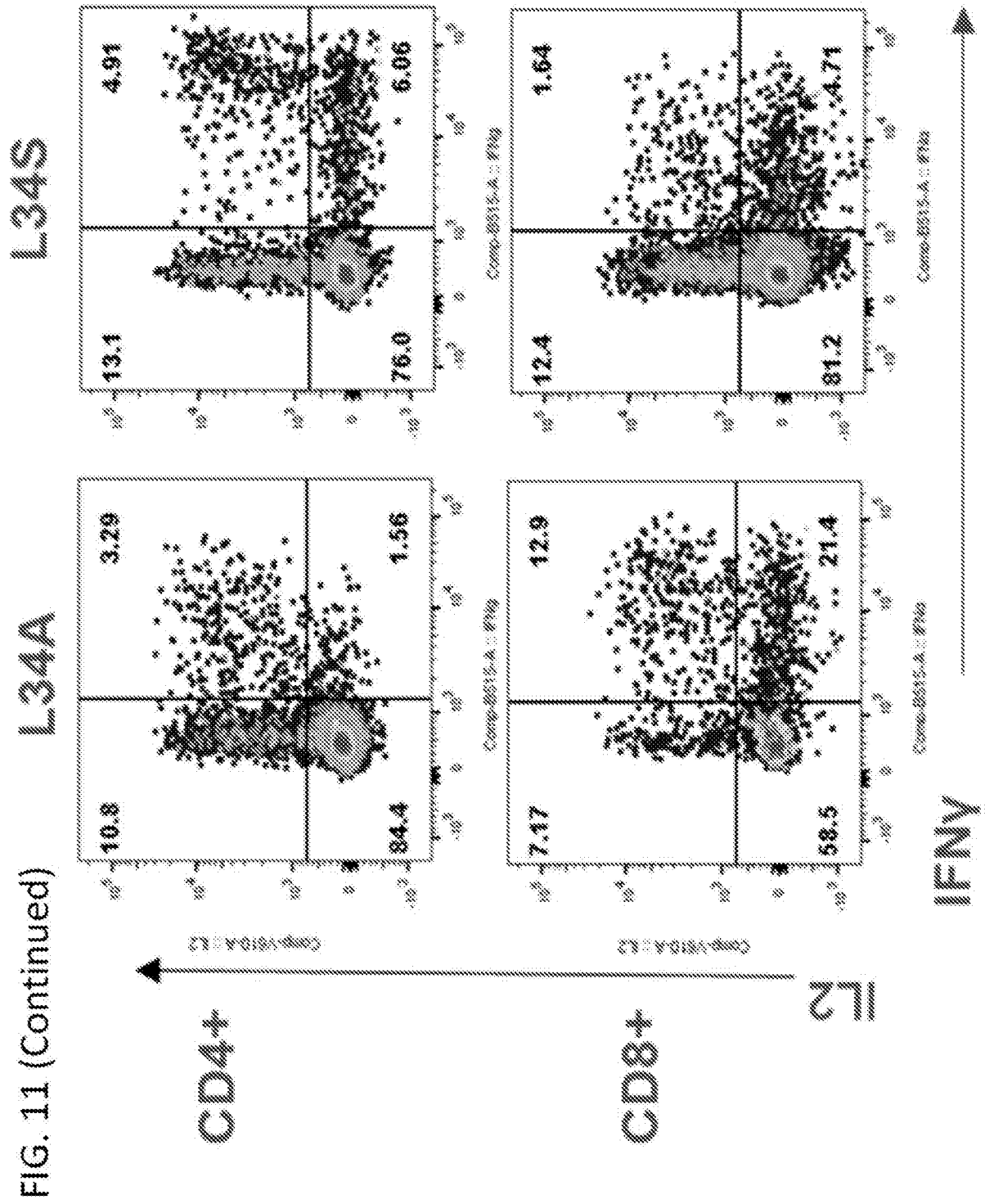
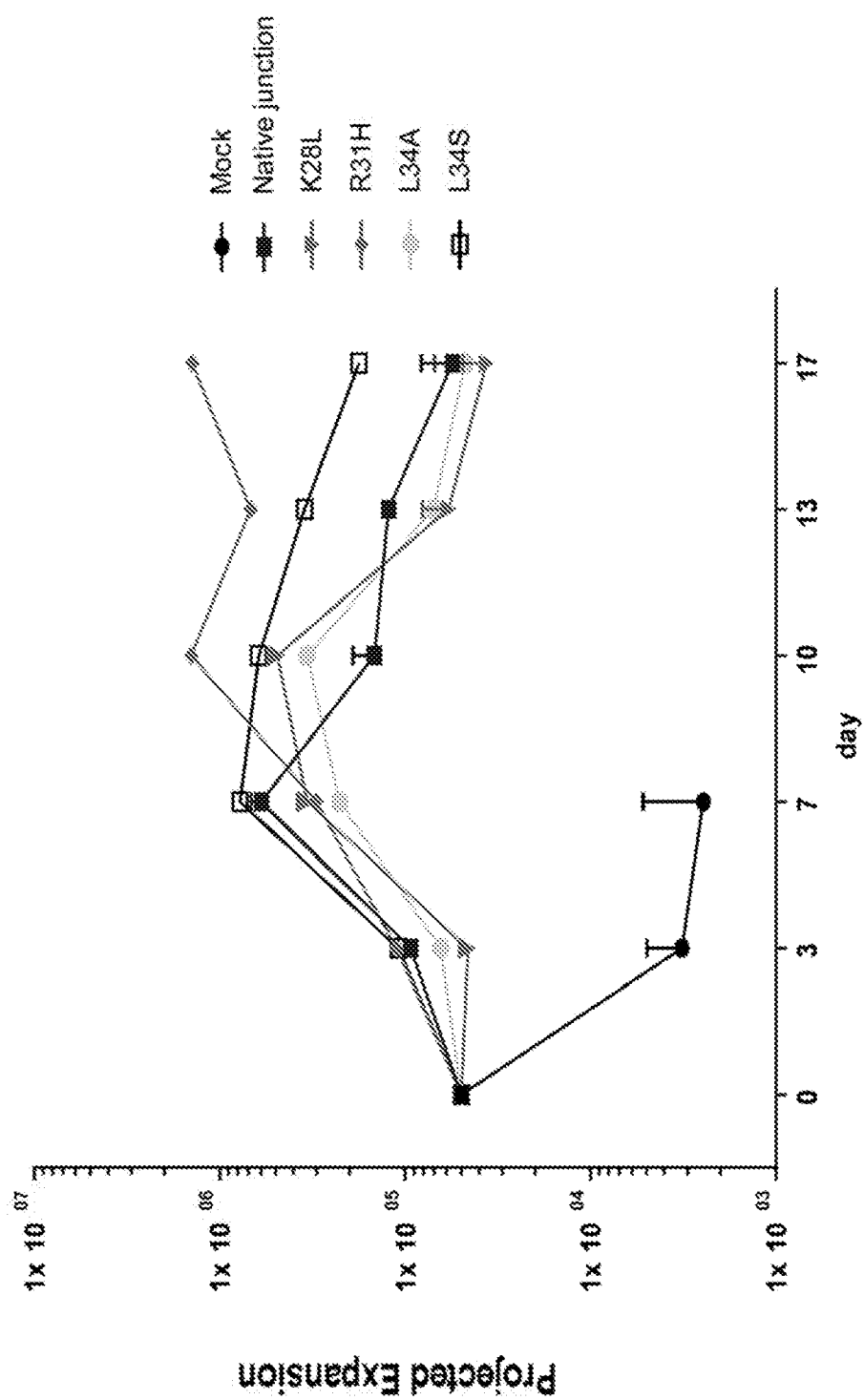


FIG. 12



INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/064861

A. CLASSIFICATION OF SUBJECT MATTER
 INV. C07K14/705
 ADD.

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)
 C07K

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/139943 A1 (CAMPANA DARIO [SG] ET AL) 21 May 2015 (2015-05-21) Example 2, SEQ ID NO:3, claims -----	17-23, 32,35, 48-73
X,P	WO 2016/090190 A1 (JUNO THERAPEUTICS INC [US]) 9 June 2016 (2016-06-09) the whole document -----	17-73
A	MARKUS CHMIELEWSKI ET AL: "Antigen-Specific T-Cell Activation Independently of the MHC: Chimeric Antigen Receptor-Redirected T Cells", FRONTIERS IN IMMUNOLOGY, vol. 4, 1 January 2013 (2013-01-01), XP055352436, DOI: 10.3389/fimmu.2013.00371 ----- -/-	17-73



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

14 March 2017

Date of mailing of the international search report

05/04/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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 Fax: (+31-70) 340-3016

Authorized officer

Gómez Ortiz, Mariola

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2016/064861

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. SADELAIN ET AL: "The Basic Principles of Chimeric Antigen Receptor Design", CANCER DISCOVERY, vol. 3, no. 4, 1 April 2013 (2013-04-01), pages 388-398, XP055287277, US ISSN: 2159-8274, DOI: 10.1158/2159-8290.CD-12-0548 -----	17-73

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/064861

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.: 1-16(completely); 17-73(partially)
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:
see FURTHER INFORMATION sheet PCT/ISA/210
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:

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 an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-16(completely); 17-73(partially)

The subject-matter of claim 1-16 is so unclear that it could not be searched :

these claims refer to a variant chimeric receptor which comprises a modified junction region with one or more modifications compared to the one of a reference chimeric receptor. Further the reference receptor is defined as comprising two domains. The junction appears to be defined as the last 15 amino acids of the first domain and/or the first 15 amino acids of the second domain and in addition a "peptide fragment" which was not mentioned before in the claim, of 8-15 amino acid of the modified junction region has a lower affinity for an HLA molecule than the corresponding fragment in the reference chimeric receptor.

The reasons for the unclearness is that there is no structural frame given to understand the context of the features meant. In addition, the claims only amount to claim the result to be achieved: a modified junction with one or more modifications which than result in lower affinity towards a HLA. Since the number of modifications is not given anything is encompassed therein.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2016/064861

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2015139943	A1	21-05-2015	NONE

WO 2016090190	A1	09-06-2016	US 2016158359 A1 09-06-2016
			WO 2016090190 A1 09-06-2016
