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(71) **Demandeur/Applicant:**
BLUEBIRD BIO, INC., US
(72) **Inventeurs/Inventors:**
CERTO, MICHAEL T., US;
LEUNG, WAI-HANG, US
(74) **Agent:** BORDEN LADNER GERVAIS LLP

(54) **Titre : COMPOSITIONS DE LYMPHOCYTES T RECEPTEURS D'ANTIGENES CHIMERIQUES**
(54) **Title: CHIMERIC ANTIGEN RECEPTOR T CELL COMPOSITIONS**

(57) **Abrégé/Abstract:**

The invention provides improved compositions for adoptive immune effector cell therapies for treatment, prevention, or amelioration of numerous conditions including, but not limited to cancer, infectious disease, autoimmune disease, inflammatory disease, and immunodeficiency.

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(71) **Applicant:** BLUEBIRD BIO, INC. [US/US]; 60 Binney Street, Cambridge, Massachusetts 02142 (US).

(72) **Inventors:** CERTO, Michael T.; 49 Fern Road, Medford, Massachusetts 02155 (US). LEUNG, Wai-Hang; 3439A 22nd Avenue W., Seattle, Washington 98199 (US).

(74) **Agents:** MASHRUWALA, Mary Anne et al.; Cooley LLP, 1299 Pennsylvania Avenue, NW, Suite 700, Washington, District of Columbia 20004 (US).

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CHIMERIC ANTIGEN RECEPTOR T CELL COMPOSITIONS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/322,547, filed April 14, 2016, and 62/319,703, filed April 7, 2016, each of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is BLBD_067_02WO_ST25.txt. The text file is 61 KB, was created on April 7, 2017, and is being submitted electronically via EFS-Web, concurrent with the filing of the specification.

BACKGROUND

Technical Field

The present invention relates to improved immune effector cell compositions for adoptive cell therapy. More particularly, the invention relates to improved chimeric antigen receptor (CAR) T cell compositions, and method of making and using the same.

Description of the Related Art

The global burden of cancer doubled between 1975 and 2000. Cancer is the second leading cause of morbidity and mortality worldwide, with approximately 14.1 million new cases and 8.2 million cancer related deaths in 2012. The most common cancers are breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, bladder cancer, melanoma of the skin, non-Hodgkin lymphoma, thyroid cancer, kidney and renal pelvis cancer,

endometrial cancer, leukemia, and pancreatic cancer. The number of new cancer cases is projected to rise to 22 million within the next two decades.

Adoptive cellular immunotherapy strategies based on the isolation, modification, expansion and reinfusion of T cells, have been explored and tested in early stage clinical trials.

5 In particular, strategies based on T cells expressing chimeric antigen receptors (CAR T cells) are fast becoming the effector cells of choice for cancer immunotherapy due to their selective recognition and powerful effector mechanisms.

However, current treatments have shown mixed rates of success, with only a small number of patients have experienced durable remissions, highlighting the as-yet unrealized
10 potential for CAR T cell-based immunotherapies.

BRIEF SUMMARY

The invention generally relates, in part, to improved immune effector cell compositions and methods of manufacturing the same. The immune effector cells contemplated in particular embodiments, comprise a chimeric antigen receptor (CAR) and a precise disruption or
15 modification in one or more T cell receptor loci, which leads to disruption of TCR expression and signaling. Surprisingly, the inventors have discovered that CAR T cells comprising one or more modified T cell receptor alpha (TCR α) alleles secrete increased amounts of cytolytic cytokines and are more therapeutically efficacious than CAR T cells that lack a modified TCR α allele.

20 In various embodiments, a chimeric antigen receptor (CAR) T cell is provided, comprising, one or more modified T cell receptor alpha (TCR α) alleles, wherein the CAR T cell secretes an increased amount of proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

25 In particular embodiments, the CAR comprises an extracellular domain that binds a target antigen selected from the group consisting of: BCMA, CD19, CSPG4, PSCA, ROR1, and TAG72.

In certain embodiments, the CAR comprises a transmembrane domain isolated from a polypeptide selected from the group consisting of: alpha or beta chain of the T-cell receptor,

CD3 δ , CD3 ϵ , CD3 γ , CD3 ζ , CD4, CD5, CD8 α , CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD154, and PD-1.

In particular embodiments, the CAR comprises one or more intracellular costimulatory signaling domains isolated from a polypeptide selected from the group consisting of: TLR1, 5 TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DAP10, LAT, NKD2C, SLP76, TRIM, and ZAP70.

In further embodiments, the CAR comprises a signaling domain isolated from a polypeptide selected from the group consisting of: FcR γ , FcR β , CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , 10 CD22, CD79a, CD79b, and CD66d.

In some embodiments, the CAR comprises a hinge region polypeptide selected from the group consisting of: a hinge region of CD8 α , a hinge region of PD1, and a hinge region of CD152.

In certain embodiments, the CAR comprises one or more linker polypeptides.

15 In particular embodiments, the CAR comprises a spacer region polypeptide that comprises CH2 and CH3 regions of IgG1, IgG4, or IgD.

In additional embodiments, the CAR comprises a signal peptide selected from the group consisting of: an IgG1 heavy chain signal polypeptide, a CD8 α signal polypeptide, and a human GM-CSF receptor alpha signal polypeptide.

20 In particular embodiments, the one or more modified TCR α alleles are non-functional or have substantially reduced function.

In further embodiments, the CAR T cell comprises one or more proviral integrants comprising a nucleic acid encoding the CAR.

In certain embodiments, the CAR T cell comprises a polynucleotide comprising a 25 promoter operably linked to a nucleic acid encoding the CAR, wherein the polynucleotide has been inserted into the one or more TCR α alleles by homology directed repair.

In additional embodiments, the one or more proinflammatory cytokines are selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α .

In various embodiments, a CAR T cell is provided, comprising: one or more modified TCR α alleles; and a nucleic acid encoding a CAR that binds a target antigen, wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when contacted with a target cell expressing the target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

In various embodiments, a CAR T cell is provided, comprising: one or more modified TCR α alleles; and one or more proviral integrants comprising a nucleic acid encoding a CAR that binds a target antigen, wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

In various embodiments, a CAR T cell is provided, comprising: one or more modified TCR α alleles, wherein a donor repair template comprising a nucleic acid encoding a CAR that binds a target antigen is inserted into the one or more TCR α alleles by homology directed repair, and wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

In certain embodiments, the nucleic acid further comprises an RNA polymerase II promoter operably linked to the polynucleotide encoding the CAR.

In some embodiments, the RNA polymerase II promoter is selected from the group consisting of: a short EF1 α promoter, a long EF1 α promoter, a human ROSA 26 locus, a Ubiquitin C (UBC) promoter, a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β -actin (CAG) promoter, a β -actin promoter and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter.

In particular embodiments, the CAR comprises an extracellular domain that binds an antigen selected from the group consisting of: BCMA, CD19, CSPG4, PSCA, ROR1, and TAG72.

In some embodiments, the CAR further comprises a transmembrane domain and one or more intracellular signaling domains.

In further embodiments, the CAR comprises a transmembrane domain, a costimulatory domain, and a primary signaling domain.

In particular embodiments, the CAR further comprises a signal peptide, one or more linker peptides, and one or more hinge peptides, and optionally one or more spacer peptides.

5 In additional embodiments, the CAR comprises an anti-BCMA scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

In certain embodiments, the CAR comprises an anti-CD19 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling
10 domain.

In some embodiments, the CAR comprises an anti-CSPG4 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

In particular embodiments, the CAR comprises an anti-ROR1 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling
15 domain.

In further embodiments, the CAR comprises an anti-PSCA scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

20 In particular embodiments, the CAR comprises an anti-TAG72 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

In additional embodiments, the one or more proinflammatory cytokines are selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-
25 1 α .

In various embodiments, a composition is provided comprising a CAR T cell contemplated herein.

In various embodiments, a composition is provided comprising a CAR T cell contemplated herein and a physiologically acceptable excipient.

In various embodiments, a method of making a CAR T cell is provided comprising: engineering a double-strand break at a target site in a TCR α allele, wherein the break is repaired by non-homologous end joining (NHEJ), thereby generating a modified TCR α allele; and transducing the T cell with a viral vector encoding a CAR; wherein the CAR T cell
 5 secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks a modified TCR α allele.

In certain embodiments, the modified TCR α allele is non-functional or has substantially reduced function.

10 In particular embodiments, the viral vector is a retroviral vector.

In some embodiments, the retroviral vector is a lentiviral vector.

In various embodiments, a method of making a CAR T cell is provided comprising: engineering a double-strand break at a target site in a TCR α allele; and transducing the T cell with a viral vector comprising a donor repair template that comprises an RNA polymerase II
 15 promoter operably linked to a nucleic acid encoding a CAR; wherein the donor repair template is incorporated into the TCR α allele by homology directed repair at the site of the double-strand break (DSB); and wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks a modified TCR α allele.

20 In certain embodiments, the donor repair template comprises a 5' homology arm homologous to the TCR α sequence 5' of the DSB; and a 3' homology arm homologous to the TCR α sequence 3' of the DSB.

In additional embodiments, the viral vector is a recombinant adeno-associated viral vector (rAAV) or a retrovirus.

25 In particular embodiments, the rAAV has one or more ITRs from AAV2.

In particular embodiments, the rAAV has a serotype selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and AAV10.

In further embodiments, the retrovirus is a lentivirus.

In some embodiments, the lentivirus is an integrase deficient lentivirus.

In particular embodiments, the double-strand break is generated with an engineered nuclease.

In certain embodiments, the engineered nuclease is selected from the group consisting of: a meganuclease, a megaTAL, a TALEN, a ZFN, or a CRISPR/Cas nuclease.

5 In certain embodiments, the meganuclease is engineered from an I-OnuI LHE.

In some embodiments, the engineered nuclease is a megaTAL comprising a TALE DNA binding domain and an engineered meganuclease.

In further embodiments, the double-strand break is generated by an engineered endonuclease and an end-processing enzyme.

10 In particular embodiments, an mRNA encoding the engineered endonuclease and an mRNA encoding the end-processing enzyme are introduced into the T cell to generate the double-strand break.

In additional embodiments, an mRNA encoding the engineered endonuclease, a viral-self cleaving peptide, and an end-processing enzyme are introduced into the T cell to generate
15 the double-strand break.

In particular embodiments, an mRNA encoding the engineered endonuclease, an IRES element, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

In further embodiments, an mRNA encoding the engineered endonuclease fused to an
20 end-processing enzyme or biologically active fragment thereof, are introduced into the T cell to generate the double-strand break.

In certain embodiments, the end-processing enzyme exhibits 5-3' exonuclease, 5-3' alkaline exonuclease, 3-5'exonuclease, 5' flap endonuclease, helicase or template-independent DNA polymerases activity.

25 In particular embodiments, the end-processing enzyme comprises Trex2 or a biologically active fragment thereof.

In some embodiments, the one or more proinflammatory cytokines are selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α .

In various embodiments, a method of treating a cancer in a subject is provided
30 comprising, administering a CAR T cell or a composition contemplated herein to the subject.

In additional embodiments, a therapeutically effective amount of CAR T cells comprising a modified TCR α allele that is administered to the subject to clear the tumor is less than the therapeutically effective amount of CAR T cells lacking a modified TCR α that is administered to the subject to clear the tumor.

5 In further embodiments, the cancer is a solid cancer.

In certain embodiments, the cancer is a liquid cancer.

In particular embodiments, the liquid cancer is a hematological malignancy selected from the group consisting of: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, hairy cell
10 leukemia (HCL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML) and polycythemia vera, Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, Burkitt lymphoma, small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, marginal zone
15 lymphoma, mycosis fungoides, anaplastic large cell lymphoma, Sézary syndrome, precursor T-lymphoblastic lymphoma, multiple myeloma, overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

In additional embodiments, the hematological malignancy is multiple myeloma.

20 In certain embodiments, the hematological malignancy is CLL.

In various embodiments, a method of treating a B cell related condition in a subject in need thereof is provided, comprising administering to the subject a therapeutically effective amount of CAR T cells or a therapeutically effective amount of a composition contemplated herein to the subject.

25 In particular embodiments, the B cell related condition is multiple myeloma, non-Hodgkin's lymphoma, B cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, post-transplant lymphoproliferative disorder, an immunoregulatory disorder, rheumatoid arthritis, myasthenia gravis, idiopathic thrombocytopenia purpura, anti-phospholipid syndrome, Chagas' disease, Grave's disease, Wegener's granulomatosis, poly-
30 arteritis nodosa, Sjogren's syndrome, pemphigus vulgaris, scleroderma, multiple sclerosis, anti-

phospholipid syndrome, ANCA associated vasculitis, Goodpasture's disease, Kawasaki disease, autoimmune hemolytic anemia, and rapidly progressive glomerulonephritis, heavy-chain disease, primary or immunocyte-associated amyloidosis, or monoclonal gammopathy of undetermined significance.

5 In certain embodiments, the B cell related condition is a B cell malignancy.

In particular embodiments, the B cell related condition is a plasma cell malignancy.

In some embodiments, the B cell related condition is an autoimmune disease.

In various embodiments, a method of increasing the efficacy of a CAR T cell is provided comprising decreasing the availability of TCR α signaling components on the CAR T
10 cell surface by introducing an engineered nuclease that creates a double-strand break (DSB) at a target site in a TCR α allele to modify the TCR α allele.

In additional embodiments, the CAR T cell comprises one or more proviral integrants encoding the CAR and wherein the DSB is repaired by non-homologous end joining (NHEJ).

In further embodiments, the CAR T cell comprises an RNA polymerase II promoter
15 operably linked to a nucleic acid encoding a CAR incorporated into the TCR α allele by homology directed repair at the DSB.

In some embodiments, the engineered nuclease is selected from the group consisting of: a meganuclease, a megaTAL, a TALEN, a ZFN, or a CRISPR/Cas nuclease.

In particular embodiments, the meganuclease is engineered from an I-OnuI LHE.

20 In further embodiments, the engineered nuclease is a megaTAL comprising a TALE DNA binding domain and an engineered meganuclease.

In certain embodiments, the double-strand break is generated by an engineered endonuclease and an end-processing enzyme.

In particular embodiments, an mRNA encoding the engineered endonuclease and an
25 mRNA encoding the end-processing enzyme are introduced into the T cell to generate the double-strand break.

In some embodiments, an mRNA encoding the engineered endonuclease, a viral-self cleaving peptide, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

In certain embodiments, an mRNA encoding the engineered endonuclease, an IRES element, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

5 In particular embodiments, an mRNA encoding the engineered endonuclease fused to an end-processing enzyme or biologically active fragment thereof, is introduced into the T cell to generate the double-strand break. In additional embodiments, the end-processing enzyme exhibits 5-3' exonuclease, 5-3' alkaline exonuclease, 3-5' exonuclease, 5' flap endonuclease, helicase or template-independent DNA polymerases activity.

10 In further embodiments, the end-processing enzyme comprises Trex2 or a biologically active fragment thereof.

In particular embodiments, the CAR T cells bound to a target antigen secrete an increased amount of one or more proinflammatory cytokines selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α , compared to a CAR T cell bound to the target antigen that lacks a modified TCR α allele.

15 BRIEF DESCRIPTION OF SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows the VCNs at day 10, for two representative donors for anti-BCMA CAR T cells cultured using the standard culture process (no EP), cells electroporated but received no mRNA (EP only), and cells electroporated with mRNA encoding a megaTAL that targets the TCR α gene (TCR α KO), both at large scale and small scale.

20 **Figure 2** shows representative growth curves over 10 days, for anti-BCMA CAR T cells cultured using the standard culture process (no EP), cells electroporated but received no mRNA (EP only), and cells electroporated with mRNA encoding a megaTAL that targets the TCR α gene (TCR α KO), both at large scale and small scale.

25 **Figure 3** shows representative FACS plots for two donors for anti-BCMA CAR expression and TCR α disruption. Anti-BCMA CAR expression was determined by staining cells with BCMA antigen conjugated to PE fluorophore. TCR α disruption was determined by anti-CD3 staining.

Figure 4 shows a graphical representation of the FACS data shown in Figure 3.

Figure 5A shows representative data for antigen dependent IFN γ release for anti-BCMA CAR T cells cultured using the standard culture process (no EP), cells electroporated but received no mRNA (EP only), and cells electroporated with mRNA encoding a megaTAL that targets the TCR α gene (TCR α KO) cultured in the presence of BCMA positive and
5 BCMA negative cell lines.

Figure 5B shows representative data for antigen dependent release of IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α for anti-BCMA CAR T cells cultured using the standard culture process (no EP), and cells electroporated with mRNA encoding a megaTAL that targets the TCR α gene (TCR α KO) +/- Trex2 cultured in the presence of BCMA positive
10 and BCMA negative cell lines.

Figure 6A shows the *in vivo* efficacy of anti-BCMA CAR T cells manufactured using the standard process (no EP), EP only, and TCR α KO treatments in a Daudi xenograft model up to day 27.

Figure 6B shows the *in vivo* efficacy of anti-BCMA CAR T cells manufactured using
15 the standard process (no EP), EP only, and TCR α KO treatments in a Daudi xenograft model up to day 43.

Figure 7 shows a representative FACS plot of anti-CD19 CAR expression in T cells transduced with lentivirus encoding an anti-CD19 CAR (LV-T cells) and T cells where an anti-CD19 CAR has been inserted into the TCR α gene using homology directed repair (HR-T
20 cells).

Figure 8A shows the *in vitro* cytotoxicity of anti-CD19 CAR T cells (LV-T cells) and anti-CD19 CAR T cells (LV-T cells) prepared from two donors in a co-culture assay with CD19 expressing Nalm-6 cells at an E:T ratio of 1:1.

Figure 8B shows representative data for antigen dependent IFN γ release for
25 untransduced control T cells and anti-CD19 CAR T cells (LV-T cells) and anti-CD19 CAR T cells (LV-T cells) prepared from two donors after 24 hours of co-culture with CD19 expressing Nalm-6 cells at an E:T ratio of 1:1.

Figure 9 shows a representative FACS plot of anti-CD19 CAR expression in T cells transduced with lentivirus encoding an anti-CD19 CAR and T cells where an anti-CD19 CAR
30 has been inserted into the TCR α gene using homology directed repair.

Figure 10A shows the *in vitro* cytotoxicity of anti-CD19 CAR T cells (LV-T cells) and anti-CD19 CAR T cells (LV-T cells) prepared from two donors in a co-culture assay with CD19 expressing Nalm-6 cells at an E:T ratio of 1:1.

Figure 10B shows representative data for antigen dependent IFN γ , IL-2, IL-4, and TNF α release for anti-CD19 CAR T cells (LV-T cells) and anti-CD19 CAR T cells (LV-T cells) prepared from two donors after 24 hours of co-culture with CD19 expressing Nalm-6 cells at an E:T ratio of 1:1.

Figure 11A shows the expression of T cell exhaustion markers for anti-CD19 CAR T cells produced by lentiviral transduction (LV-CAR T cells) or homologous recombination HR-CAR T cells) cultured in the presence of CD19 expressing Nalm-6 cells for 24 hours.

Figure 11B shows the expression of T cell exhaustion markers for anti-CD19 CAR T cells produced by lentiviral transduction (LV-CAR T cells) or homologous recombination HR-CAR T cells) cultured in the presence of CD19 expressing Nalm-6 cells for 72 hours.

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

SEQ ID NO: 1 sets forth the polynucleotide sequence of I-OnuI.

SEQ ID NO: 2 sets forth the polypeptide sequence encoded by SEQ ID NO: 1.

SEQ ID NOs: 3 and 4 set forth illustrative examples of TCR α target sites for genome editing.

SEQ ID NOs: 5-7 set forth polypeptide sequences of engineered I-OnuI variants.

SEQ ID NO: 8 sets forth the polypeptide sequence of an anti-CD19 CAR.

SEQ ID NO: 9 sets forth the polynucleotide sequence of plasmid, pBW1021.

SEQ ID NO: 10 sets forth the TCR α I-OnuI megaTAL target site.

SEQ ID NO: 11 sets forth the polypeptide sequence of an illustrative example of a TCR α I-OnuI megaTAL.

SEQ ID NO: 12 sets forth the polypeptide sequence of an illustrative example of an anti-BCMA CAR.

SEQ ID NO: 13 sets forth the polynucleotide sequence of plasmid, pBW400.

SEQ ID NOs: 14-24 set forth the amino acid sequences of various linkers.

SEQ ID NOs: 25-49 set forth the amino acid sequences of protease cleavage sites and self-cleaving polypeptide cleavage sites.

DETAILED DESCRIPTION

A. OVERVIEW

5 The invention generally relates, in part, to improved immune effector cell compositions and methods of manufacturing the same. The immune effector cells contemplated in particular embodiments, comprise a chimeric antigen receptor (CAR) and a precise disruption or modification in one or more T cell receptor loci, which leads to disruption of TCR expression and signaling. Surprisingly, the inventors have discovered that genome edited CAR T cells
10 comprising one or more modified TCR α alleles manufactured by the methods contemplated herein are imbued with increased therapeutic efficacy, e.g., increased antigen dependent secretion of proinflammatory cytokines, e.g., IFN γ , TNF α , IL-2, and other molecules including cytotoxins, e.g., Perforin, Granzyme A, Granzyme B, that increase the cytolytic activity of CAR T cells toward tumor cells.

15 In various embodiments, CAR T cells comprise a modification of one or more alleles of the T cell receptor alpha (TCR α) gene. In particular embodiments, modification of one or more TCR α alleles ablates or substantially ablates expression of the TCR α allele(s), decreases expression of the TCR α allele(s), and/or impairs, substantially impairs, or ablates one or more functions of the TCR α allele(s) or renders the TCR α allele(s) non-functional. In particular
20 embodiments, TCR α functions include, but are not limited to, recruiting CD3 to the cell surface, MHC dependent recognition and binding of antigen, activation of TCR $\alpha\beta$ signaling.

In various embodiments, wherein a DNA break is generated in the TCR α gene of a cell, NHEJ of the ends of the cleaved genomic sequence may result in a cell with normal TCR expression, expression of a loss-of- or gain-of-function TCR, or preferably, a cell that lacks
25 functional TCR expression, e.g., lacks the ability to recruit CD3 to cell surface, activate TCR $\alpha\beta$ signaling, recognize and bind MHC-antigen complexes. In one preferred embodiment, the repair is biased toward NHEJ and loss-of-function by using engineered endonucleases and an

end-processing enzyme, *e.g.*, 3' to 5' exonuclease (Trex2) or biologically active fragment thereof.

In various other embodiments, wherein a donor template for repair of the cleaved TCR α genomic sequence is provided, a TCR α allele is repaired with the sequence of the
5 template by homologous recombination at the DNA break-site. In preferred embodiments, the repair template comprises a polynucleotide sequence that encodes a CAR.

Accordingly, the methods and compositions contemplated herein represent a quantum improvement compared to existing adoptive cell therapies.

The practice of the particular embodiments will employ, unless indicated specifically to
10 the contrary, conventional methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, and cell biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. *See e.g.*, Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual* (3rd Edition, 2001); Sambrook, *et al.*, *Molecular*
15 *Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982); Ausubel *et al.*, *Current Protocols in Molecular Biology* (John Wiley and Sons, updated July 2008); *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, Greene Pub. Associates and Wiley-Interscience; Glover, *DNA Cloning: A Practical Approach*, vol. I & II (IRL Press, Oxford,
20 1985); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); *Transcription and Translation* (B. Hames & S. Higgins, Eds., 1984); Perbal, *A Practical Guide to Molecular Cloning* (1984); Harlow and Lane, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998) *Current Protocols in Immunology* Q. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, eds., 1991);
25 *Annual Review of Immunology*; as well as monographs in journals such as *Advances in Immunology*.

B. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention

belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of particular embodiments, preferred embodiments of compositions, methods and materials are described herein. For the purposes of the present disclosure, the following terms are defined below.

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

 The use of the alternative (*e.g.*, “or”) should be understood to mean either one, both, or any combination thereof of the alternatives.

10 The term “and/or” should be understood to mean either one, or both of the alternatives.

 As used herein, the term “about” or “approximately” refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In one
15 embodiment, the term “about” or “approximately” refers a range of quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length $\pm 15\%$, $\pm 10\%$, $\pm 9\%$, $\pm 8\%$, $\pm 7\%$, $\pm 6\%$, $\pm 5\%$, $\pm 4\%$, $\pm 3\%$, $\pm 2\%$, or $\pm 1\%$ about a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length.

 In one embodiment, a range, *e.g.*, 1 to 5, about 1 to 5, or about 1 to about 5, refers to
20 each numerical value encompassed by the range. For example, in one non-limiting and merely illustrative embodiment, the range “1 to 5” is equivalent to the expression 1, 2, 3, 4, 5; or 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0; or 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0.

25 Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By “consisting of” is meant including, and limited to, whatever follows the phrase “consisting of.” Thus, the phrase “consisting of” indicates that the
30 listed elements are required or mandatory, and that no other elements may be present. By

“consisting essentially of” is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase “consisting essentially of” indicates that the listed elements are required or mandatory, but that no other elements are present that materially
 5 affect the activity or action of the listed elements.

Reference throughout this specification to “one embodiment,” “an embodiment,” “a particular embodiment,” “a related embodiment,” “a certain embodiment,” “an additional embodiment,” or “a further embodiment” or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in
 10 at least one embodiment. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. It is also understood that the positive recitation of a feature in one embodiment, serves as a basis for excluding the feature in a particular
 15 embodiment.

An “immune effector cell,” is any cell of the immune system that has one or more effector functions (*e.g.*, cytotoxic cell killing activity, secretion of cytokines, induction of ADCC and/or CDC). Illustrative immune effector cells contemplated in particular
 20 embodiments are T lymphocytes, in particular cytotoxic T cells (CTLs; CD8⁺ T cells), TILs, and helper T cells (HTLs; CD4⁺ T cells). In one embodiment, immune effector cells include natural killer (NK) cells. In one embodiment, immune effector cells include natural killer T (NKT) cells. In preferred embodiments, the immune effector cell is a CAR T cell.

The terms “T cell” or “T lymphocyte” are art-recognized and are intended to include thymocytes, naïve T lymphocytes, immature T lymphocytes, mature T lymphocytes, resting T
 25 lymphocytes, or activated T lymphocytes. A T cell can be a T helper (Th) cell, for example a T helper 1 (Th1) or a T helper 2 (Th2) cell. The T cell can be a helper T cell (HTL; CD4⁺ T cell) CD4⁺ T cell, a cytotoxic T cell (CTL; CD8⁺ T cell), a tumor infiltrating cytotoxic T cell (TIL; CD8⁺ T cell), CD4⁺CD8⁺ T cell, CD4⁺CD8⁻ T cell, or any other subset of T cells. In one embodiment, the T cell is an NKT cell. Other illustrative populations of T cells suitable for use

in particular embodiments include naïve T cells and memory T cells. In preferred embodiments, T cells are modified to express a CAR.

“Potent T cells,” and “young T cells,” are used interchangeably in particular embodiments and refer to T cell phenotypes wherein the T cell is capable of proliferation and a
5 concomitant decrease in differentiation. In particular embodiments, the young T cell has the phenotype of a “naïve T cell.” In particular embodiments, young T cells comprise one or more of, or all of the following biological markers: CD62L, CCR7, CD28, CD27, CD122, CD127, CD197, and CD38. In one embodiment, young T cells comprise one or more of, or all of the following biological markers: CD62L, CD127, CD197, and CD38. In one embodiment, the
10 young T cells lack expression of CD57, CD244, CD160, PD-1, CTLA4, TIM3, and LAG3.

As used herein, the term “proliferation” refers to an increase in cell division, either symmetric or asymmetric division of cells. In particular embodiments, “proliferation” refers to the symmetric or asymmetric division of T cells. “Increased proliferation” occurs when there is an increase in the number of cells in a treated sample compared to cells in a non-treated
15 sample.

As used herein, the term “differentiation” refers to a method of decreasing the potency or proliferation of a cell or moving the cell to a more developmentally restricted state. In particular embodiments, differentiated T cells acquire immune effector cell functions.

As used herein, the terms “T cell manufacturing” or “methods of manufacturing T
20 cells’ or comparable terms refer to the process of producing a therapeutic composition of T cells, which manufacturing methods may comprise one or more of, or all of the following steps: harvesting, stimulation, activation, genome editing, and expansion.

The term “*ex vivo*” refers generally to activities that take place outside an organism, such as experimentation or measurements done in or on living tissue in an artificial
25 environment outside the organism, preferably with minimum alteration of the natural conditions. In particular embodiments, “*ex vivo*” procedures involve living cells or tissues taken from an organism and cultured or modulated in a laboratory apparatus, usually under sterile conditions, and typically for a few hours or up to about 24 hours, but including up to 48 or 72 hours, depending on the circumstances. In certain embodiments, such tissues or cells can
30 be collected and frozen, and later thawed for *ex vivo* treatment. Tissue culture experiments or

procedures lasting longer than a few days using living cells or tissue are typically considered to be “*in vitro*,” though in certain embodiments, this term can be used interchangeably with *ex vivo*.

The term “*in vivo*” refers generally to activities that take place inside an organism, such as cell self-renewal and cell proliferation or expansion. In one embodiment, the term “*in vivo* expansion” refers to the ability of a cell population to increase in number *in vivo*. In one embodiment, cells are engineered or modified *in vivo*.

The term “stimulation” refers to a primary response induced by binding of a stimulatory molecule (*e.g.*, a TCR/CD3 complex) with its cognate ligand thereby mediating a signal transduction event including, but not limited to, signal transduction via the TCR/CD3 complex.

A “stimulatory molecule,” refers to a molecule on a T cell that specifically binds with a cognate stimulatory ligand.

A “stimulatory ligand,” as used herein, means a ligand that when present on an antigen presenting cell (*e.g.*, an aAPC, a dendritic cell, a B-cell, and the like) can specifically bind with a cognate binding partner (referred to herein as a “stimulatory molecule”) on a T cell, thereby mediating a primary response by the T cell, including, but not limited to, activation, initiation of an immune response, proliferation, and the like. Stimulatory ligands include, but are not limited to CD3 ligands, *e.g.*, an anti-CD3 antibody and CD2 ligands, *e.g.*, anti-CD2 antibody, and peptides, *e.g.*, CMV, HPV, EBV peptides.

The term, “activation” refers to the state of a T cell that has been sufficiently stimulated to induce detectable cellular proliferation. In particular embodiments, activation can also be associated with induced cytokine production, and detectable effector functions. The term “activated T cells” refers to, among other things, T cells that are proliferating. Signals generated through the TCR alone are insufficient for full activation of the T cell and one or more secondary or costimulatory signals are also required. Thus, T cell activation comprises a primary stimulation signal through the TCR/CD3 complex and one or more secondary costimulatory signals. Co-stimulation can be evidenced by proliferation and/or cytokine production by T cells that have received a primary activation signal, such as stimulation through the CD3/TCR complex or through CD2.

A “costimulatory signal,” refers to a signal, which in combination with a primary signal, such as TCR/CD3 ligation, leads to T cell proliferation, cytokine production, and/or upregulation or downregulation of particular molecules (*e.g.*, CD28).

5 A “costimulatory ligand,” refers to a molecule that binds a costimulatory molecule. A costimulatory ligand may be soluble or provided on a surface. A “costimulatory molecule” refers to the cognate binding partner on a T cell that specifically binds with a costimulatory ligand (*e.g.*, anti-CD28 antibody).

“Autologous,” as used herein, refers to cells where the donor and recipient are the same subject.

10 “Allogeneic,” as used herein, refers to cells wherein the donor and recipient species are the same but the cells are genetically different.

“Syngeneic,” as used herein, refers to cells wherein the donor and recipient species are the same, the donor and recipient are different individuals, and the donor cells and recipient cells are genetically identical.

15 “Xenogeneic,” as used herein, refers to cells wherein the donor and recipient species are different.

As used herein, the terms “individual” and “subject” are often used interchangeably and refer to any animal that exhibits a symptom of cancer or other immune disorder that can be treated with the gene therapy vectors, cell-based therapeutics, and methods contemplated
20 elsewhere herein. Suitable subjects (*e.g.*, patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included. Typical subjects include human patients that have, have been diagnosed with, or are at risk or having, cancer or another immune disorder.

25 As used herein, the term “patient” refers to a subject that has been diagnosed with cancer or another immune disorder that can be treated with the compositions and methods disclosed elsewhere herein.

As used herein “treatment” or “treating,” includes any beneficial or desirable effect on the symptoms or pathology of a disease or pathological condition, and may include even
30 minimal reductions in one or more measurable markers of the disease or condition being

treated, *e.g.*, cancer, autoimmune disease, immune disorder, *etc.* Treatment can optionally involve delaying of the progression of the disease or condition. “Treatment” does not necessarily indicate complete eradication or cure of the disease or condition, or associated symptoms thereof.

5 As used herein, “prevent,” and similar words such as “prevention,” “prevented,” “preventing” *etc.*, indicate an approach for preventing, inhibiting, or reducing the likelihood of the occurrence or recurrence of, a disease or condition, *e.g.*, cancer, autoimmune disease, immune disorder, *etc.* It also refers to delaying the onset or recurrence of a disease or condition or delaying the occurrence or recurrence of the symptoms of a disease or condition. As used
10 herein, “prevention” and similar words also includes reducing the intensity, effect, symptoms and/or burden of a disease or condition prior to onset or recurrence of the disease or condition.

As used herein, the phrase “ameliorating at least one symptom of” refers to decreasing one or more symptoms of the disease or condition for which the subject is being treated, *e.g.*, cancer, infectious disease, autoimmune disease, inflammatory disease, and immunodeficiency.
15 In particular embodiments, the disease or condition being treated is a cancer, wherein the one or more symptoms ameliorated include, but are not limited to, weakness, fatigue, shortness of breath, easy bruising and bleeding, frequent infections, enlarged lymph nodes, distended or painful abdomen (due to enlarged abdominal organs), bone or joint pain, fractures, unplanned weight loss, poor appetite, night sweats, persistent mild fever, and decreased urination (due to
20 impaired kidney function).

As used herein, the term “amount” refers to “an amount effective” or “an effective amount” of genome edited CAR T cells sufficient to achieve a beneficial or desired prophylactic or therapeutic result, including clinical results.

A “prophylactically effective amount” refers to an amount of genome edited CAR T
25 cells effective 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 is less than the therapeutically effective amount.

A “therapeutically effective amount” of genome edited CAR T cells may vary according to factors such as the disease state, age, sex, and weight of the individual, and the
30 ability of the genome edited CAR T cell to elicit a desired response in the individual. A

therapeutically effective amount is also one in which any toxic or detrimental effects of the virus or transduced therapeutic cells are outweighed by the therapeutically beneficial effects. The term “therapeutically effective amount” includes an amount that is effective to “treat” a subject (*e.g.*, a patient). When a therapeutic amount is indicated, the precise amount of the
5 compositions contemplated in particular embodiments, to be administered, can be determined by a physician in view of the specification and with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject).

An “immune disorder” refers to a disease that evokes a response from the immune system. In particular embodiments, the term “immune disorder” refers to a cancer, an
10 autoimmune disease, or an immunodeficiency. In one embodiment, immune disorders encompasses infectious disease.

As used herein, the term “cancer” relates generally to a class of diseases or conditions in which abnormal cells divide without control and can invade nearby tissues.

As used herein, the term “malignant” refers to a cancer in which a group of tumor cells
15 display one or more of uncontrolled growth (*i.e.*, division beyond normal limits), invasion (*i.e.*, intrusion on and destruction of adjacent tissues), and metastasis (*i.e.*, spread to other locations in the body via lymph or blood).

As used herein, the term “metastasize” refers to the spread of cancer from one part of the body to another. A tumor formed by cells that have spread is called a “metastatic tumor” or
20 a “metastasis.” The metastatic tumor contains cells that are like those in the original (primary) tumor.

As used herein, the term “benign” or “non-malignant” refers to tumors that may grow larger but do not spread to other parts of the body. Benign tumors are self-limited and typically do not invade or metastasize.

25 A “cancer cell” or “tumor cell” refers to an individual cell of a cancerous growth or tissue. A tumor refers generally to a swelling or lesion formed by an abnormal growth of cells, which may be benign, pre-malignant, or malignant. Most cancers form tumors, but some, *e.g.*, leukemia, do not necessarily form tumors. For those cancers that form tumors, the terms cancer (cell) and tumor (cell) are used interchangeably. The amount of a tumor in an

individual is the “tumor burden” which can be measured as the number, volume, or weight of the tumor.

An “autoimmune disease” refers to a disease in which the body produces an immunogenic (*i.e.*, immune system) response to some constituent of its own tissue. In other words, the immune system loses its ability to recognize some tissue or system within the body as “self” and targets and attacks it as if it were foreign. Autoimmune diseases can be classified into those in which predominantly one organ is affected (*e.g.*, hemolytic anemia and anti-immune thyroiditis), and those in which the autoimmune disease process is diffused through many tissues (*e.g.*, systemic lupus erythematosus). For example, multiple sclerosis is thought to be caused by T cells attacking the sheaths that surround the nerve fibers of the brain and spinal cord. This results in loss of coordination, weakness, and blurred vision. Autoimmune diseases are known in the art and include, for instance, Hashimoto’s thyroiditis, Grave’s disease, lupus, multiple sclerosis, rheumatic arthritis, hemolytic anemia, anti-immune thyroiditis, systemic lupus erythematosus, celiac disease, Crohn’s disease, colitis, diabetes, scleroderma, psoriasis, and the like.

An “immunodeficiency” means the state of a patient whose immune system has been compromised by disease or by administration of chemicals. This condition makes the system deficient in the number and type of blood cells needed to defend against a foreign substance. Immunodeficiency conditions or diseases are known in the art and include, for example, AIDS (acquired immunodeficiency syndrome), SCID (severe combined immunodeficiency disease), selective IgA deficiency, common variable immunodeficiency, X-linked agammaglobulinemia, chronic granulomatous disease, hyper-IgM syndrome, and diabetes.

An “infectious disease” refers to a disease that can be transmitted from person to person or from organism to organism, and is caused by a microbial or viral agent (*e.g.*, common cold). Infectious diseases are known in the art and include, for example, hepatitis, sexually transmitted diseases (*e.g.*, Chlamydia, gonorrhea), tuberculosis, HIV/AIDS, diphtheria, hepatitis B, hepatitis C, cholera, and influenza.

By “enhance” or “promote” or “increase” or “expand” or “potentiate” refers generally to the ability of a composition contemplated herein, *e.g.*, genome edited CAR T cells comprising one or more modified TCR α alleles, to produce, elicit, or cause a greater response

(*i.e.*, physiological response) compared to the response caused by CAR T cells expressing the same or substantially the same CAR, but where the TCR α alleles have not been modified (control composition). A measurable response may include an increase proinflammatory cytokine release, *e.g.*, IFN γ , increases in cytolytic activity, or an increase in CAR expression, among other measurable responses apparent from the understanding in the art and the description herein. An “increased” or “enhanced” amount is typically a “statistically significant” amount, and may include an increase that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.*, 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.*, 1.5, 1.6, 1.7, 1.8, *etc.*) the response produced by a vehicle or control composition.

By “decrease” or “lower” or “lessen” or “reduce” or “abate” or “ablate” or “inhibit” or “dampen” refers generally to the ability of composition contemplated herein to produce, elicit, or cause a lesser response (*i.e.*, physiological response) compared to the response caused by either vehicle or a control molecule/composition. A measurable response may include a decrease in endogenous TCR expression or function, and the like. A “decrease” or “reduced” amount is typically a “statistically significant” amount, and may include a decrease that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.*, 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.*, 1.5, 1.6, 1.7, 1.8, *etc.*) the response (reference response) produced by a vehicle or control composition.

By “maintain,” or “preserve,” or “maintenance,” or “no change,” or “no substantial change,” or “no substantial decrease” refers generally to the ability of a composition contemplated herein to produce, elicit, or cause a substantially similar or comparable physiological response (*i.e.*, downstream effects) in a cell, as compared to the response caused by either vehicle, a control molecule/composition, or the response in a particular cell lineage. A comparable response is one that is not significantly different or measurable different from the reference response.

The terms “specific binding affinity” or “specifically binds” or “specifically bound” or “specific binding” or “specifically targets” as used herein, describe binding of one molecule to another at greater binding affinity than background binding. A binding domain “specifically binds” to a target molecule if it binds to or associates with a target molecule with an affinity or

K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of $1/M$) of, for example, greater than or equal to about $10^5 M^{-1}$. In certain embodiments, a binding domain binds to a target with a K_a greater than or equal to about $10^6 M^{-1}$, $10^7 M^{-1}$, $10^8 M^{-1}$, $10^9 M^{-1}$, $10^{10} M^{-1}$, $10^{11} M^{-1}$, $10^{12} M^{-1}$, or $10^{13} M^{-1}$. “High affinity” binding domains refers to those binding domains with a K_a of at least $10^7 M^{-1}$, at least $10^8 M^{-1}$, at least $10^9 M^{-1}$, at least $10^{10} M^{-1}$, at least $10^{11} M^{-1}$, at least $10^{12} M^{-1}$, at least $10^{13} M^{-1}$, or greater.

Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, $10^{-5} M$ to $10^{-13} M$, or less). Affinities of binding domain polypeptides contemplated in particular embodiments can be readily determined using conventional techniques, *e.g.*, by competitive ELISA (enzyme-linked immunosorbent assay), or by binding association, or displacement assays using labeled ligands, or using a surface-plasmon resonance device such as the Biacore T100, which is available from Biacore, Inc., Piscataway, NJ, or optical biosensor technology such as the EPIC system or EnSpire that are available from Corning and Perkin Elmer respectively (*see also, e.g.*, Scatchard *et al.* (1949) *Ann. N.Y. Acad. Sci.* 51:660; and U.S. Patent Nos. 5,283,173; 5,468,614, or the equivalent).

In one embodiment, the affinity of specific binding is about 2 times greater than background binding, about 5 times greater than background binding, about 10 times greater than background binding, about 20 times greater than background binding, about 50 times greater than background binding, about 100 times greater than background binding, or about 1000 times greater than background binding or more.

An “antigen (Ag)” refers to a compound, composition, or substance, *e.g.*, lipid, carbohydrate, polysaccharide, glycoprotein, peptide, or nucleic acid, that can stimulate the production of antibodies or a T cell response in an animal, including compositions (such as one that includes a tumor-specific protein) that are injected or absorbed into an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous antigens, such as the disclosed antigens. A “target antigen” or “target antigen or interest” is an antigen that a binding domain of an engineered antigen receptor contemplated herein, is designed to bind. In one embodiment, the antigen is an MHC-peptide complex, such as a class I MHC-peptide complex or a class II MHC-peptide complex.

An “epitope” or “antigenic determinant” refers to the region of an antigen to which a binding agent binds.

As used herein, “isolated polynucleotide” refers to a polynucleotide that has been purified from the sequences which flank it in a naturally-occurring state, *e.g.*, a DNA fragment
5 that has been removed from the sequences that are normally adjacent to the fragment. An “isolated polynucleotide” also refers to a complementary DNA (cDNA), a recombinant DNA, or other polynucleotide that does not exist in nature and that has been made by the hand of man.

An “isolated protein,” “isolated peptide,” or “isolated polypeptide” and the like, as used
10 herein, refer to *in vitro* synthesis, isolation, and/or purification of a peptide or polypeptide molecule from a cellular environment, and from association with other components of the cell, *i.e.*, it is not significantly associated with *in vivo* substances.

An “isolated cell” refers to a non-naturally occurring cell, *e.g.*, a T cell that does not exist in nature, a modified T cell, an engineered T cell, *etc.*, that has been obtained from an *in*
15 *vivo* tissue or organ and is substantially free of extracellular matrix.

“Recombination” refers to a process of exchange of genetic information between two polynucleotides, including but not limited to, donor capture by non-homologous end joining (NHEJ) and homologous recombination. For the purposes of this disclosure, “homologous recombination (HR)” refers to the specialized form of such exchange that takes place, for
20 example, during repair of double-strand breaks in cells via homology-directed repair (HDR) mechanisms. This process requires nucleotide sequence homology, uses a “donor” molecule as a template to repair a “target” molecule (*i.e.*, the one that experienced the double-strand break), and is variously known as “non-crossover gene conversion” or “short tract gene conversion,” because it leads to the transfer of genetic information from the donor to the target. Without
25 wishing to be bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or “synthesis-dependent strand annealing,” in which the donor is used to resynthesize genetic information that will become part of the target, and/or related processes. Such specialized HR often results in an alteration of the sequence of the target molecule such that part or all of the sequence of
30 the donor polynucleotide is incorporated into the target polynucleotide.

“Cleavage” refers to the breakage of the covalent backbone of a DNA molecule. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible. Double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, polypeptides contemplated herein are used for targeted double-stranded DNA cleavage.

A “target site” or “target sequence” is a chromosomal or extrachromosomal nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind and/or cleave, provided sufficient conditions for binding and/or cleavage exist.

An “exogenous” molecule is a molecule that is not normally present in a cell, but that is introduced into a cell by one or more genetic, biochemical or other methods. Exemplary exogenous molecules include, but are not limited to small organic molecules, protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, biopolymer nanoparticle, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer.

An “endogenous” molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, endogenous TCRs.

A “gene,” refers to a DNA region encoding a gene product, as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. A gene includes, but is not limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding

sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

“Gene expression” refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

As used herein, the term “genome editing” refers to the substitution, deletion, and/or introduction of genetic material at a target site in the cell’s genome, which restores, corrects, and/or modifies expression of a gene. Genome editing contemplated in particular embodiments comprises introducing one or more engineered nucleases into a cell to generate DNA lesions at a target site in the cell’s genome, optionally in the presence of a donor repair template.

As used herein, the term “genetically engineered” or “genetically modified” refers to the chromosomal or extrachromosomal addition of extra genetic material in the form of DNA or RNA to the total genetic material in a cell. Genetic modifications may be targeted or non-targeted to a particular site in a cell’s genome. In one embodiment, genetic modification is site specific. In one embodiment, genetic modification is not site specific.

C. GENOME EDITED CAR T CELLS

The genome edited cells manufactured by the methods contemplated in particular embodiments comprise improved CAR T cell compositions. In one embodiment, a CAR T cell comprises one or more modified TCR α alleles. Surprisingly, the inventors have discovered that genome edited CAR T cells comprising one or more modified TCR α alleles manufactured by the methods contemplated herein are imbued with increased therapeutic efficacy, *e.g.*, increased antigen dependent secretion of proinflammatory cytokines, *e.g.*, IFN γ , TNF α , IL-2, and other molecules including cytotoxins, *e.g.*, Perforin, Granzyme A, Granzyme B, that increase the cytolytic activity of CAR T cells toward tumor cells. Without wishing to be bound

to any particular theory, it is contemplated that increased CAR T cell efficacy is due, in part, to decreased TCR $\alpha\beta$ receptor expression freeing up accessory signaling components for CARs, increased cytokine secretion, and reduction of tonic signaling.

In various embodiments, genome edited CAR T cells comprise one or more TCR α alleles modified by the gene editing compositions and methods contemplated herein.

In one embodiment, genome edited CAR T cells comprise one or more modified TCR α alleles; and a nucleic acid encoding a CAR.

In one embodiment, genome edited CAR T cells comprise one or more modified TCR α alleles; and one or more proviral integrants comprising a nucleic acid encoding a CAR. In one embodiment, the proviral integrants are retroviral- or lentiviral-based proviral integrants.

In one embodiment, genome edited CAR T cells comprise one or more modified TCR α alleles; wherein the one or more TCR α alleles have been modified by inserting a donor repair template comprising a nucleic acid encoding a CAR into the one or more TCR α alleles by homology directed repair.

In particular embodiments, a method of making a CAR T cell comprising one or more modified TCR α alleles comprises activating a population of T cells and stimulating the population of T cells to proliferate; introducing an engineered nuclease into the population of T cells to generate a double-strand break at a TCR α allele; and introducing a nucleic acid encoding a chimeric antigen receptor (CAR) into the T cell.

In certain embodiments, a method of making a CAR T cell comprising one or more modified TCR α alleles comprises activating a population of T cells and stimulating the population of T cells to proliferate; and transducing the T cell with a viral vector encoding a CAR. Expression of the engineered nuclease creates a double-strand break at a target site in the TCR α allele that is preferentially repaired by NHEJ. In one embodiment, an engineered endonuclease is introduced into the cell with a 3' end-processing enzyme, *e.g.*, a 3' to 5' exonuclease such as Trex2, to increase the bias to repair the double-strand break by NHEJ. In one embodiment, the T cell is transduced with a retroviral vector, *e.g.*, gamma retroviral vector or lentiviral vector, encoding a CAR. In one embodiment, the T cell is transduced with a lentiviral vector encoding a CAR.

In some embodiments, a method of making a CAR T cell comprising one or more modified TCR α alleles comprises activating a population of T cells and stimulating the population of T cells to proliferate; and transducing the T cell with a viral vector comprising a donor repair template that comprises a nucleic acid encoding a CAR; wherein expression of the engineered nuclease creates a double-strand break at a target site in the TCR α allele, and the donor repair template is incorporated into the TCR α allele by homology directed repair (HDR) at the site of the double-strand break (DSB). In one embodiment, the donor template further comprises an RNAPol II promoter operably linked to a nucleic acid encoding a CAR.

Genome edited CAR T cells contemplated in particular embodiments may be autologous/autogeneic (“self”) or non-autologous (“non-self,” *e.g.*, allogeneic, syngeneic or xenogeneic). “Autologous,” as used herein, refers to cells from the same subject. “Allogeneic,” as used herein, refers to cells of the same species that differ genetically to the cell in comparison. “Syngeneic,” as used herein, refers to cells of a different subject that are genetically identical to the cell in comparison. “Xenogeneic,” as used herein, refers to cells of a different species to the cell in comparison. In preferred embodiments, the T cells are obtained from a mammalian subject. In a more preferred embodiment, the T cells are obtained from a primate subject. In the most preferred embodiment, the T cells are obtained from a human subject.

T cells can be obtained from a number of sources including, but not limited to, peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled person, such as sedimentation, *e.g.*, FICOLL™ separation.

In other embodiments, an isolated or purified population of T cells is used. In some embodiments, after isolation of PBMC, both cytotoxic and helper T lymphocytes can be sorted into naïve, memory, and effector T cell subpopulations either before or after activation, expansion, and/or genetic modification.

A specific subpopulation of T cells, expressing one or more of the following markers: CD3, CD4, CD8, CD28, CD45RA, CD45RO, CD62, CD127, and HLA-DR can be further

isolated by positive or negative selection techniques. In one embodiment, a specific subpopulation of T cells, expressing one or more of the markers selected from the group consisting of CD62L, CCR7, CD28, CD27, CD122, CD127, CD197; or CD38 or CD62L, CD127, CD197, and CD38, is further isolated by positive or negative selection techniques. In
5 various embodiments, the manufactured T cell compositions do not express or do not substantially express one or more of the following markers: CD57, CD244, CD160, PD-1, CTLA4, TIM3, and LAG3.

In one embodiment, an isolated or purified population of T cells expresses one or more of the markers including, but not limited to a CD3⁺, CD4⁺, CD8⁺, or a combination thereof

10 In certain embodiments, the T cells are isolated from an individual and first activated and stimulated to proliferate *in vitro* prior to undergoing genome editing.

In order to achieve sufficient therapeutic doses of T cell compositions, T cells are often subjected to one or more rounds of stimulation, activation and/or expansion. T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents
15 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; and 6,867,041, each of which is incorporated herein by reference in its entirety. In particular embodiments, T cells are activated and expanded for about 1 day to about 4 days, about 1 day to about 3 days, about 1 day to about 2 days, about 2 days to about 3 days, about 2 days to about 4 days, about 3
20 days to about 4 days, or about 1 day, about 2 days, about 3 days, or about 4 days prior to introduction of the genome editing compositions into the T cells.

In particular embodiments, T cells are activated and expanded for about 6 hours, about 12 hours, about 18 hours or about 24 hours prior to introduction of the genome editing compositions into the T cells.

25 In one embodiment, T cells are activated at the same time that genome editing compositions are introduced into the T cells.

In one embodiment, a costimulatory ligand is presented on an antigen presenting cell (*e.g.*, an aAPC, dendritic cell, B cell, and the like) that specifically binds a cognate costimulatory molecule on a T cell, thereby providing a signal which, in addition to the primary
30 signal provided by, for instance, binding of a TCR/CD3 complex, mediates a desired T cell

response. Suitable costimulatory ligands include, but are not limited to, CD7, B7-1 (CD80), B7-2 (CD86), 4-1BBL, OX40L, inducible costimulatory ligand (ICOS-L), intercellular adhesion molecule (ICAM), CD30L, CD40, CD70, CD83, HLA-G, MICA, MICB, lymphotoxin beta receptor, ILT3, ILT4, an agonist or antibody that binds Toll ligand receptor, and a ligand that specifically binds with B7-H3.

In a particular embodiment, a costimulatory ligand comprises an antibody or antigen binding fragment thereof that specifically binds to a costimulatory molecule present on a T cell, including but not limited to, CD27, CD28, 4- IBB, OX40, CD30, CD40, ICOS, lymphocyte function-associated antigen- 1 (LFA-1), CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83.

Suitable costimulatory ligands further include target antigens, which may be provided in soluble form or expressed on APCs or aAPCs that bind engineered antigen receptors expressed on genome edited T cells.

In various embodiments, a method of generating a genome edited CAR T cell comprises activating a population of cells comprising T cells and expanding the population of T cells. T cell activation can be accomplished by providing a primary stimulation signal through the T cell TCR/CD3 complex and by providing a secondary costimulation signal through an accessory molecule, *e.g.*, CD28.

The TCR/CD3 complex may be stimulated by contacting the T cell with a suitable CD3 binding agent, *e.g.*, a CD3 ligand or an anti-CD3 monoclonal antibody. Illustrative examples of CD3 antibodies include, but are not limited to, OKT3, G19-4, BC3, and 64.1.

In addition to the primary stimulation signal provided through the TCR/CD3 complex, induction of T cell responses requires a second, costimulatory signal. In particular embodiments, a CD28 binding agent can be used to provide a costimulatory signal. Illustrative examples of CD28 binding agents include but are not limited to: natural CD 28 ligands, *e.g.*, a natural ligand for CD28 (*e.g.*, a member of the B7 family of proteins, such as B7-1(CD80) and B7-2 (CD86); and anti-CD28 monoclonal antibody or fragment thereof capable of crosslinking the CD28 molecule, *e.g.*, monoclonal antibodies 9.3, B-T3, XR-CD28, KOLT-2, 15E8, 248.23.2, and EX5.3D10.

In one embodiment, the molecule providing the primary stimulation signal, for example a molecule which provides stimulation through the TCR/CD3 complex and the costimulatory molecule are coupled to the same surface.

5 In certain embodiments, binding agents that provide stimulatory and costimulatory signals are localized on the surface of a cell. This can be accomplished by transfecting or transducing a cell with a nucleic acid encoding the binding agent in a form suitable for its expression on the cell surface or alternatively by coupling a binding agent to the cell surface.

In another embodiment, the molecule providing the primary stimulation signal, for example a molecule which provides stimulation through the TCR/CD3 complex and the
10 costimulatory molecule are displayed on antigen presenting cells.

In one embodiment, the molecule providing the primary stimulation signal, for example a molecule which provides stimulation through the TCR/CD3 complex and the costimulatory molecule are provided on separate surfaces.

In a certain embodiment, one of the binding agents that provides stimulatory and
15 costimulatory signals is soluble (provided in solution) and the other agent(s) is provided on one or more surfaces.

In a particular embodiment, the binding agents that provide stimulatory and costimulatory signals are both provided in a soluble form (provided in solution).

In various embodiments, the methods for making genome edited CAR T cells
20 contemplated herein comprise activating T cells with anti-CD3 and anti-CD28 antibodies.

In one embodiment, expanding T cells activated by the methods contemplated herein further comprises culturing a population of cells comprising T cells for several hours (about 3 hours) to about 7 days to about 28 days or any hourly integer value in between. In another embodiment, the T cell composition may be cultured for 14 days. In a particular embodiment,
25 T cells are cultured for about 21 days. In another embodiment, the T cell compositions are cultured for about 2-3 days. Several cycles of stimulation/activation/expansion may also be desired such that culture time of T cells can be 60 days or more.

In particular embodiments, conditions appropriate for T cell culture include an appropriate media (*e.g.*, Minimal Essential Media or RPMI Media 1640 or, X-vivo 15,
30 (Lonza)) and one or more factors necessary for proliferation and viability including, but not

limited to serum (*e.g.*, fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, IL-21, GM-CSF, IL-10, IL-12, IL-15, TGF β , and TNF- α or any other additives suitable for the growth of cells known to the skilled artisan.

Further illustrative examples of cell culture media include, but are not limited to RPMI
5 1640, Clicks, AIM-V, DMEM, MEM, a-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells.

Antibiotics, *e.g.*, penicillin and streptomycin, are included only in experimental
10 cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (*e.g.*, 37° C) and atmosphere (*e.g.*, air plus 5% CO₂).

In particular embodiments, PBMCs or isolated T cells are contacted with a stimulatory agent and costimulatory agent, such as anti-CD3 and anti-CD28 antibodies, generally attached
15 to a bead or other surface, in a culture medium with appropriate cytokines, such as IL-2, IL-7, and/or IL-15.

In other embodiments, artificial APC (aAPC) made by engineering K562, U937, 721.221, T2, and C1R cells to direct the stable expression and secretion, of a variety of costimulatory molecules and cytokines. In a particular embodiment K32 or U32 aAPCs are
20 used to direct the display of one or more antibody-based stimulatory molecules on the aAPC cell surface. Populations of T cells can be expanded by aAPCs expressing a variety of costimulatory molecules including, but not limited to, CD137L (4-1BBL), CD134L (OX40L), and/or CD80 or CD86. Finally, the aAPCs provide an efficient platform to expand genetically modified T cells and to maintain CD28 expression on CD8 T cells. aAPCs provided in WO
25 03/057171 and US2003/0147869 are hereby incorporated by reference in their entirety.

In various embodiments, a method for generating a CAR T cell comprising a modified TCR α allele comprises introducing one or more engineered nucleases contemplated herein into the population of T cells to generate a double-strand break in a TCR α allele.

In one embodiment, the one or more nucleases contemplated herein are introduced into
30 the T cell prior to activation and stimulation.

In another embodiment, the one or more nucleases contemplated herein are introduced into the T cell at about the same time that the T cell is stimulated.

In a preferred embodiment, the one or more nucleases contemplated herein are introduced into the T cell after the T cell activation and stimulation, *e.g.*, about 1, 2, 3, or 4 days after. The nucleases introduced into the T cells in particular embodiments, include, but are not limited to an endonuclease, *e.g.*, a meganuclease, a megaTAL, a TALEN, a ZFN, or a CRISPR/Cas nuclease; and optionally an end-processing nuclease or biologically active fragment thereof, *e.g.*, 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity.

The endonuclease and end-processing nuclease may be expressed as a fusion protein, may be expressed from a polycistronic mRNA, or independently expressed from one or more mRNAs or expression cassettes.

In particular embodiments, the one or more nucleases are introduced into a T cell using a vector. In other embodiments, the one or more nucleases are preferably introduced into a T cell as mRNAs. The nucleases may be introduced into the T cells by microinjection, transfection, lipofection, heat-shock, electroporation, transduction, gene gun, microinjection, DEAE-dextran-mediated transfer, and the like.

Genome editing methods contemplated in particular embodiments comprise introducing one or more engineered nucleases contemplated herein into a population of activated and stimulated T cells in order to create a DSB at a target site that is preferentially repaired via NHEJ, and subsequently transducing the population of T cells with a vector encoding a CAR.

Genome editing methods contemplated in particular embodiments comprise introducing one or more engineered nucleases contemplated herein into a population of activated and stimulated T cells in order to create a DSB at a target site and optionally, subsequently introducing one or more donor repair templates into the population of T cells that will be incorporated into the cell's genome at the DSB site by homologous recombination.

In a particular embodiment, one or more donor templates comprising a polynucleotide encoding a CAR are introduced into the population of T cells. The donor templates may be introduced into the T cells by microinjection, transfection, lipofection, heat-shock,

electroporation, transduction, gene gun, microinjection, DEAE-dextran-mediated transfer, and the like.

In a preferred embodiment, the one or more nucleases are introduced into the T cell by mRNA electroporation and the one or more donor repair templates are introduced into the T
5 cell by viral transduction.

In another preferred embodiment, the one or more nucleases are introduced into the T cell by mRNA electroporation and the one or more donor repair templates are introduced into the T cell by AAV transduction. The AAV vector may comprise ITRs from AAV2, and a serotype from any one of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8,
10 AAV9, or AAV10. In preferred embodiments, the AAV vector may comprise ITRs from AAV2 and a serotype from AAV6.

In particular embodiments, the donor repair template comprises one or more homology arms. As used herein, the term “homology arms” refers to a nucleic acid sequence in a donor template that is identical, or nearly identical, to the DNA sequence flanking the DNA break
15 introduced by the nuclease at a target site. In one embodiment, the donor template comprises a 5′ homology arm that comprises a nucleic acid that is identical or nearly identical to the DNA sequence 5′ of the DNA break site. In one embodiment, the donor template comprises a 3′ homology arm that comprises a nucleic acid that is identical or nearly identical to the DNA sequence 3′ of the DNA break site. In a preferred embodiment, the donor template comprises a
20 5′ homology arm and a 3′ homology arm.

Illustrative examples of suitable lengths of homology arms contemplated in particular embodiments, may be independently selected, and include but are not limited to: about 100 bp, about 200 bp, about 300 bp, about 400 bp, about 500 bp, about 600 bp, about 700 bp, about 800 bp, about 900 bp, about 1000 bp, about 1100 bp, about 1200 bp, about 1300 bp, about
25 1400 bp, about 1500 bp, about 1600 bp, about 1700 bp, about 1800 bp, about 1900 bp, about 2000 bp, about 2100 bp, about 2200 bp, about 2300 bp, about 2400 bp, about 2500 bp, about 2600 bp, about 2700 bp, about 2800 bp, about 2900 bp, or about 3000 bp, or longer homology arms, including all intervening lengths of homology arms.

Additional illustrative examples of suitable homology arm lengths include, but are not
30 limited to: about 100 bp to about 3000 bp, about 200 bp to about 3000 bp, about 300 bp to

about 3000 bp, about 400 bp to about 3000 bp, about 500 bp to about 3000 bp, about 500 bp to about 2500 bp, about 500 bp to about 2000 bp, about 750 bp to about 2000 bp, about 750 bp to about 1500 bp, or about 1000 bp to about 1500 bp, including all intervening lengths of homology arms.

5 In a particular embodiment, the lengths of the 5' and 3' homology arms are independently selected from about 500 bp to about 1500 bp. In one embodiment, the 5' homology arm is about 1500 bp and the 3' homology arm is about 1000 bp. In one embodiment, the 5' homology arm is about 600 bp and the 3' homology arm is about 600 bp.

Donor repair templates may further comprises one or more polynucleotides such as
10 promoters and/or enhancers, untranslated regions (UTRs), Kozak sequences, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, internal ribosomal entry sites (IRES), recombinase recognition sites (*e.g.*, LoxP, FRT, and Att sites), termination codons, transcriptional termination signals, and polynucleotides encoding self-cleaving polypeptides, epitope tags, contemplated elsewhere herein.

15 In various embodiments, the donor repair template comprises a 5' homology arm, an RNA polymerase II promoter, a CAR, and a 3' homology arm.

In another preferred embodiment, the one or more nucleases are introduced into the T cell by mRNA electroporation and the one or more donor repair templates are introduced into the T cell by lentiviral transduction. The lentiviral vector backbone may be derived from HIV-
20 1, HIV-2, visna-maedi virus (VMV) virus, caprine arthritis-encephalitis virus (CAEV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV), bovine immune deficiency virus (BIV), or simian immunodeficiency virus (SIV). The lentiviral vector may be integration competent or an integrase deficient lentiviral vector (IDLV). In one embodiment, IDLV vectors comprising an HIV-based vector backbone (*i.e.*, HIV cis-acting sequence
25 elements) are preferred.

The one or more donor repair templates may be delivered prior to, simultaneously with, or after the one or more engineered nucleases are introduced into a cell. In certain embodiments, the one or more donor repair templates are delivered simultaneously with the one or more engineered nucleases. In other embodiments, the one or more donor repair
30 templates are delivered prior to the one or more engineered nucleases, for example, seconds to

hours to days before the one or more donor repair templates, including, but not limited to about 1 min. to about 30 min., about 1 min. to about 60 min., about 1 min. to about 90 min., about 1 hour to about 24 hours before the one or more engineered nucleases or more than 24 hours before the one or more engineered nucleases. In certain embodiments, the one or more donor repair templates are delivered after the nuclease, preferably within about 1, 2, 3, 4, 5, 6, 7, or 8 hours; more preferably, within about 1, 2, 3, or 4 hours; or more preferably, within about 4 hours.

The one or more donor repair templates may be delivered using the same delivery systems as the one or more engineered nucleases. By way of non-limiting example, when delivered simultaneously, the donor repair templates and engineered nucleases may be encoded by the same vector, *e.g.*, an IDLV lentiviral vector or an AAV vector (*e.g.*, AAV6). In particular preferred embodiments, the engineered nuclease(s) are delivered by mRNA electroporation and the donor repair templates are delivered by transduction with an AAV vector.

In particular embodiments, methods of generating CAR T cells with one or more modified TCR α alleles comprises contacting the cells with a stimulatory agent and costimulatory agent, such as soluble anti-CD3 and anti-CD28 antibodies, or antibodies attached to a bead or other surface, in a culture medium with appropriate cytokines, such as IL-2, IL-7, and/or IL-15 and/or one or more agents that modulate a PI3K cell signaling pathway.

As used herein, the term “PI3K inhibitor” refers to a nucleic acid, peptide, compound, or small organic molecule that binds to and inhibits at least one activity of PI3K. The PI3K proteins can be divided into three classes, class 1 PI3Ks, class 2 PI3Ks, and class 3 PI3Ks. Class 1 PI3Ks exist as heterodimers consisting of one of four p110 catalytic subunits (p110 α , p110 β , p110 δ , and p110 γ) and one of two families of regulatory subunits. In particular embodiments, a PI3K inhibitor targets the class 1 PI3K inhibitors. In one embodiment, a PI3K inhibitor will display selectivity for one or more isoforms of the class 1 PI3K inhibitors (*i.e.*, selectivity for p110 α , p110 β , p110 δ , and p110 γ or one or more of p110 α , p110 β , p110 δ , and p110 γ). In another aspect, a PI3K inhibitor will not display isoform selectivity and be considered a “pan-PI3K inhibitor.” In one embodiment, a PI3K inhibitor will compete for binding with ATP to the PI3K catalytic domain.

Illustrative examples of PI3K inhibitors suitable for use particular embodiments include, but are not limited to, BKM120 (class 1 PI3K inhibitor, Novartis), XL147 (class 1 PI3K inhibitor, Exelixis), (pan-PI3K inhibitor, GlaxoSmithKline), and PX-866 (class 1 PI3K inhibitor; p110 α , p110 β , and p110 γ isoforms, Oncothyreon).

5 Other illustrative examples of selective PI3K inhibitors include, but are not limited to BYL719, GSK2636771, TGX-221, AS25242, CAL-101, ZSTK474, and IPI-145.

Further illustrative examples of pan-PI3K inhibitors include, but are not limited to BEZ235, LY294002, GSK1059615, TG100713, and GDC-0941.

In a preferred embodiment, the PI3K inhibitor is ZSTK474.

10 D. NUCLEASES

Immune effector cell compositions contemplated in particular embodiments are generated by genome editing accomplished with engineered nucleases targeting one or more TCR α alleles.

In various embodiments, CAR T cells are edited using engineered nucleases, designed
15 to bind and cleave a target DNA sequence in the T cell receptor alpha (TCR α) gene. The engineered nucleases contemplated in particular embodiments, can be used to introduce a double-strand break in a target polynucleotide sequence, which may be repaired by non-homologous end joining (NHEJ) in the absence of a polynucleotide template, *e.g.*, a donor repair template, and subsequently transduced with a virus encoding a CAR; or by homology
20 directed repair (HDR), *i.e.*, homologous recombination, in the presence of a donor repair template encoding a CAR. Engineered nucleases contemplated in certain embodiments, can also be engineered as nickases, which generate single-stranded DNA breaks that can be repaired using the cell's base-excision-repair (BER) machinery or homologous recombination in the presence of a donor repair template. NHEJ is an error-prone process that frequently
25 results in the formation of small insertions and deletions that disrupt gene function.

Homologous recombination requires homologous DNA as a template for repair and can be leveraged to create a limitless variety of modifications specified by the introduction of donor DNA containing the desired sequence at the target site, flanked on either side by sequences bearing homology to regions flanking the target site.

An “engineered nuclease” refers to a nuclease comprising one or more DNA binding domains and one or more DNA cleavage domains, wherein the nuclease has been designed and/or modified to bind a DNA binding target sequence adjacent to a DNA cleavage target sequence. The engineered nuclease may be designed and/or modified from a naturally occurring nuclease or from a previously engineered nuclease. Engineered nucleases contemplated in particular embodiments may further comprise one or more additional functional domains, *e.g.*, an end-processing enzymatic domain of an end-processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity. In particular embodiments, engineered HEs are introduced into a T cell with an end-processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity. The HE and 3' processing enzyme may be introduced separately, *e.g.*, in different vectors or separate mRNAs, or together, *e.g.*, as a fusion protein, or in a polycistronic construct separated by a viral self-cleaving peptide or an IRES element.

Illustrative examples of nucleases that may be engineered to bind and cleave a target sequence include, but are not limited to homing endonucleases (meganucleases), megaTALs, transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly-interspaced short palindromic repeats (CRISPR)/Cas nuclease systems.

In particular embodiments, the nucleases contemplated herein comprise one or more heterologous DNA-binding and cleavage domains (*e.g.*, ZFNs, TALENs, megaTALs), (Boissel *et al.*, 2014; Christian *et al.*, 2010). In other embodiments, the DNA-binding domain of a naturally-occurring nuclease may be altered to bind to a selected target site (*e.g.*, a meganuclease that has been engineered to bind to site different than the cognate binding site). For example, meganucleases have been designed to bind target sites different from their cognate binding sites (Boissel *et al.*, 2014). In particular embodiments, a nuclease requires a nucleic acid sequence to target the nuclease to a target site (*e.g.*, CRISPR/Cas).

In various embodiments, a homing endonuclease or meganuclease is engineered to bind to, and to introduce single-stranded nicks or double-strand breaks (DSBs) in a TCR α locus. “Homing endonuclease” and “meganuclease” are used interchangeably and refer to naturally-

occurring nucleases or engineered meganucleases that recognize 12-45 base-pair cleavage sites and are commonly grouped into five families based on sequence and structure motifs:

LAGLIDADG, GIY-YIG, HNH, His-Cys box, and PD-(D/E)XK.

Engineered HEs do not exist in nature and can be obtained by recombinant DNA
5 technology or by random mutagenesis. Engineered HEs may be obtained by making one or more amino acid alterations, *e.g.*, mutating, substituting, adding, or deleting one or more amino acids, in a naturally occurring HE or previously engineered HE. In particular embodiments, an engineered HE comprises one or more amino acid alterations to the DNA recognition interface.

A “DNA recognition interface” refers to the HE amino acid residues that interact with
10 nucleic acid target bases as well as those residues that are adjacent. For each HE, the DNA recognition interface comprises an extensive network of side chain-to-side chain and side chain-to-DNA contacts, most of which is necessarily unique to recognize a particular nucleic acid target sequence. Thus, the amino acid sequence of the DNA recognition interface corresponding to a particular nucleic acid sequence varies significantly and is a feature of any
15 natural or engineered HE. By way of non-limiting example, an engineered HE contemplated in particular embodiments may be derived by constructing libraries of HE variants in which one or more amino acid residues localized in the DNA recognition interface of the natural HE (or a previously engineered HE) are varied. The libraries may be screened for target cleavage activity against each predicted TCR α locus target sites using cleavage assays (*see e.g.*, Jarjour
20 *et al.*, 2009. *Nuc. Acids Res.* **37**(20): 6871-6880).

LAGLIDADG homing endonucleases (LHE) are the most well studied family of meganucleases, are primarily encoded in archaea and in organellar DNA in green algae and fungi, and display the highest overall DNA recognition specificity. LHEs comprise one or two
25 LAGLIDADG catalytic motifs per protein chain and function as homodimers or single chain monomers, respectively. Structural studies of LAGLIDADG proteins identified a highly conserved core structure (Stoddard 2005), characterized by an $\alpha\beta\beta\alpha\beta\beta\alpha$ fold, with the LAGLIDADG motif belonging to the first helix of this fold. The highly efficient and specific cleavage of LHE’s represent a protein scaffold to derive novel, highly specific endonucleases. However, engineering LHEs to bind and cleave a non-natural or non-canonical target site
30 requires selection of the appropriate LHE scaffold, examination of the target locus, selection of

putative target sites, and extensive alteration of the LHE to alter its DNA contact points and cleavage specificity, at up to two-thirds of the base-pair positions in a target site.

Illustrative examples of LHEs from which engineered LHEs may be designed include, but are not limited to I-AabMI, I-AaeMI, I-AniI, I-ApaMI, I-CapIII, I-CapIV, I-CkaMI, I-CpaMI, I-CpaMII, I-CpaMIII, I-CpaMIV, I-CpaMV, I-CpaV, I-CraMI, I-EjeMI, I-GpeMI, I-GpiI, I-GzeMI, I-GzeMII, I-GzeMIII, I-HjeMI, I-LtrII, I-LtrI, I-LtrWI, I-MpeMI, I-MveMI, I-NcrII, I-NcrI, I-NcrMI, I-OheMI, I-OnuI, I-OsoMI, I-OsoMII, I-OsoMIII, I-OsoMIV, I-PanMI, I-PanMII, I-PanMIII, I-PnoMI, I-ScuMI, I-SmaMI, I-SscMI, and I-Vdi141I.

In one embodiment, the engineered LHE is selected from the group consisting of: I-CpaMI, I-HjeMI, I-OnuI, I-PanMI, and SmaMI.

In one embodiment, the engineered LHE is I-OnuI. *See e.g.*, SEQ ID NOs: 1 and 2.

In one embodiment, engineered I-OnuI LHEs targeting the human TCR α gene were generated from a natural I-OnuI. In a preferred embodiment, engineered I-OnuI LHEs targeting the human TCR α gene were generated from a previously engineered I-OnuI. In one embodiment, engineered I-OnuI LHEs were generated against a human TCR α gene target site set forth in SEQ ID NO: 3. In one embodiment, engineered I-OnuI LHEs were generated against a human TCR α gene target site set forth in SEQ ID NO: 4.

In a particular embodiment, the engineered I-OnuI LHE comprises one or more amino acid substitutions in the DNA recognition interface. In particular embodiments, the I-OnuI LHE comprises at least 70%, at least 71%, at least 72%, at least 73%, at least 74%, at least 75%, at least 76%, at least 77%, at least 78%, at least 79%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity with the DNA recognition interface of I-OnuI (Taekuchi *et al.* 2011. *Proc Natl Acad Sci U. S. A.* 2011 Aug 9; 108(32): 13077-13082) or an engineered variant of I-OnuI as set forth in SEQ ID NOs: 5, 6, or 7, or further engineered variants thereof.

In one embodiment, the I-OnuI LHE comprises at least 70%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 95%, more preferably at least 97%, more preferably at least 99% sequence identity with the DNA

recognition interface of I-OnuI (Taekuchi *et al.* 2011. *Proc Natl Acad Sci U. S. A.* 2011 Aug 9; 108(32): 13077-13082) or an engineered variant of I-OnuI as set forth in SEQ ID NOs: 5, 6, or 7, or further engineered variants thereof.

In a particular embodiment, an engineered I-OnuI LHE comprises one or more amino acid substitutions or modifications in the DNA recognition interface, particularly in the subdomains situated from positions 24-50, 68 to 82, 180 to 203 and 223 to 240 of I-OnuI (SEQ ID NO: 2) or an engineered variant of I-OnuI as set forth in SEQ ID NOs: 5, 6, or 7, or further engineered variants thereof.

In one embodiment, an engineered I-OnuI LHE comprises one or more amino acid substitutions or modifications at additional positions situated anywhere within the entire I-OnuI sequence. The residues which may be substituted and/or modified include but are not limited to amino acids that contact the nucleic acid target or that interact with the nucleic acid backbone or with the nucleotide bases, directly or via a water molecule. In one non-limiting example an engineered I-OnuI LHE contemplated herein comprises one or more substitutions and/or modifications, preferably at least 5, preferably at least 10, preferably at least 15, more preferably at least 20, even more preferably at least 25 in at least one position selected from the position group consisting of positions: 19, 24, 26, 28, 30, 32, 34, 35, 36, 37, 38, 40, 42, 44, 46, 48, 68, 70, 72, 75, 76 77, 78, 80, 82, 168, 180, 182, 184, 186, 188, 189, 190, 191, 192, 193, 195, 197, 199, 201, 203, 223, 225, 227, 229, 231, 232, 234, 236, 238, 240 of I-OnuI (SEQ ID NO: 2) or an engineered variant of I-OnuI as set forth in SEQ ID NOs: 5, 6, or 7, or further engineered variants thereof.

In a particular embodiment, an engineered I-OnuI LHE contemplated herein comprises one or more amino acids substitutions and/or modifications selected from the group consisting of: L26I, R28D, N32R, K34N, S35E, V37N, G38R, S40R, E42S, G44R, V68K, A70T, N75R, S78M, K80R, L138M, S159P, E178D, C180Y, F182G, I186K, S188V, S190G, K191N, L192A, G193K, Q195Y, Q197G, V199R, T203S, K207R, Y223S, K225W, and D236E.

In one embodiment, the I-OnuI LHE has an amino acid sequence as set forth in SEQ ID NOs: 5, 6, or 7, or further engineered variants thereof.

Various illustrative embodiments contemplate a megaTAL nuclease that binds to and cleaves a target region of a TCR α locus. A “megaTAL” refers to an engineered nuclease

comprising an engineered TALE DNA binding domain and an engineered meganuclease, and optionally comprise one or more linkers and/or additional functional domains, *e.g.*, an end-processing enzymatic domain of an end-processing enzyme that exhibits 5-3' exonuclease, 5-3' alkaline exonuclease, 3-5'exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity. In particular embodiments, a megaTAL can be introduced into a T cell with an end-processing enzyme that exhibits 5-3' exonuclease, 5-3' alkaline exonuclease, 3-5'exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity. The megaTAL and 3' processing enzyme may be introduced separately, *e.g.*, in different vectors or separate mRNAs, or together, *e.g.*, as a fusion protein, or in a polycistronic construct separated by a viral self-cleaving peptide or an IRES element.

A "TALE DNA binding domain" is the DNA binding portion of transcription activator-like effectors (TALE or TAL-effectors), which mimics plant transcriptional activators to manipulate the plant transcriptome (*see e.g.*, Kay *et al.*, 2007. *Science* 318:648-651). TALE DNA binding domains contemplated in particular embodiments are engineered *de novo* or from naturally occurring TALEs, *e.g.*, AvrBs3 from *Xanthomonas campestris pv. vesicatoria*, *Xanthomonas gardneri*, *Xanthomonas translucens*, *Xanthomonas axonopodis*, *Xanthomonas perforans*, *Xanthomonas alfalfa*, *Xanthomonas citri*, *Xanthomonas euvesicatoria*, and *Xanthomonas oryzae* and brg11 and hpx17 from *Ralstonia solanacearum*. Illustrative examples of TALE proteins for deriving and designing DNA binding domains are disclosed in U.S. Patent No. 9,017,967, and references cited therein, all of which are incorporated herein by reference in their entireties.

In particular embodiments, a megaTAL comprises a TALE DNA binding domain comprising one or more repeat units that are involved in binding of the TALE DNA binding domain to its corresponding target DNA sequence. A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length. Each TALE DNA binding domain repeat unit includes 1 or 2 DNA-binding residues making up the Repeat Variable Di-Residue (RVD), typically at positions 12 and/or 13 of the repeat. The natural (canonical) code for DNA recognition of these TALE DNA binding domains has been determined such that an HD sequence at positions 12 and 13 leads to a binding to cytosine (C), NG binds to T, NI to A, NN

binds to G or A, and NG binds to T. In certain embodiments, non-canonical (atypical) RVDs are contemplated.

Illustrative examples of non-canonical RVDs suitable for use in particular megaTALs contemplated in particular embodiments include, but are not limited to HH, KH, NH, NK, NQ, 5 RH, RN, SS, NN, SN, KN for recognition of guanine (G); NI, KI, RI, HI, SI for recognition of adenine (A); NG, HG, KG, RG for recognition of thymine (T); RD, SD, HD, ND, KD, YG for recognition of cytosine (C); NV, HN for recognition of A or G; and H*, HA, KA, N*, NA, NC, NS, RA, S* for recognition of A or T or G or C, wherein (*) means that the amino acid at position 13 is absent. Additional illustrative examples of RVDs suitable for use in particular 10 megaTALs contemplated in particular embodiments further include those disclosed in U.S. Patent No. 8,614,092, which is incorporated herein by reference in its entirety.

In particular embodiments, a megaTAL contemplated herein comprises a TALE DNA binding domain comprising 3 to 30 repeat units. In certain embodiments, a megaTAL comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 15 28, 29, or 30 TALE DNA binding domain repeat units. In a preferred embodiment, a megaTAL contemplated herein comprises a TALE DNA binding domain comprising 5-13 repeat units, more preferably 7-12 repeat units, more preferably 9-11 repeat units, and more preferably 9, 10, or 11 repeat units.

In particular embodiments, a megaTAL contemplated herein comprises a TALE DNA 20 binding domain comprising 3 to 30 repeat units and an additional single truncated TALE repeat unit comprising 20 amino acids located at the C-terminus of a set of TALE repeat units, *i.e.*, an additional C-terminal half-TALE DNA binding domain repeat unit (amino acids -20 to -1 of the C-cap disclosed elsewhere herein, *infra*). Thus, in particular embodiments, a megaTAL contemplated herein comprises a TALE DNA binding domain comprising 3.5 to 30.5 repeat 25 units. In certain embodiments, a megaTAL comprises 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5, 19.5, 20.5, 21.5, 22.5, 23.5, 24.5, 25.5, 26.5, 27.5, 28.5, 29.5, or 30.5 TALE DNA binding domain repeat units. In a preferred embodiment, a megaTAL contemplated herein comprises a TALE DNA binding domain comprising 5.5-13.5 repeat units, more preferably 7.5-12.5 repeat units, more preferably 9.5-11.5 repeat units, and 30 more preferably 9.5, 10.5, or 11.5 repeat units.

In particular embodiments, a megaTAL comprises an “N-terminal domain (NTD)” polypeptide, one or more TALE repeat domains/units, a “C-terminal domain (CTD)” polypeptide, and an engineered meganuclease.

As used herein, the term “N-terminal domain (NTD)” polypeptide refers to the sequence that flanks the N-terminal portion or fragment of a naturally occurring TALE DNA binding domain. The NTD sequence, if present, may be of any length as long as the TALE DNA binding domain repeat units retain the ability to bind DNA. In particular embodiments, the NTD polypeptide comprises at least 120 to at least 140 or more amino acids N-terminal to the TALE DNA binding domain (0 is amino acid 1 of the most N-terminal repeat unit). In particular embodiments, the NTD polypeptide comprises at least about 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, or at least 140 amino acids N-terminal to the TALE DNA binding domain. In one embodiment, a megaTAL contemplated herein comprises an NTD polypeptide of at least about amino acids +1 to +122 to at least about +1 to +137 of a *Xanthomonas* TALE protein (0 is amino acid 1 of the most N-terminal repeat unit). In particular embodiments, the NTD polypeptide comprises at least about 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, or 137 amino acids N-terminal to the TALE DNA binding domain of a *Xanthomonas* TALE protein. In one embodiment, a megaTAL contemplated herein comprises an NTD polypeptide of at least amino acids +1 to +121 of a *Ralstonia* TALE protein (0 is amino acid 1 of the most N-terminal repeat unit). In particular embodiments, the NTD polypeptide comprises at least about 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, or 137 amino acids N-terminal to the TALE DNA binding domain of a *Ralstonia* TALE protein.

As used herein, the term “C-terminal domain (CTD)” polypeptide refers to the sequence that flanks the C-terminal portion or fragment of a naturally occurring TALE DNA binding domain. The CTD sequence, if present, may be of any length as long as the TALE DNA binding domain repeat units retain the ability to bind DNA. In particular embodiments, the CTD polypeptide comprises at least 20 to at least 85 or more amino acids C-terminal to the last full repeat of the TALE DNA binding domain (the first 20 amino acids are the half-repeat unit C-terminal to the last C-terminal full repeat unit). In particular embodiments, the CTD polypeptide comprises at least about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,

35, 36, 37, 38, 39, 40, 41, 42, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, or at least 85 amino acids C-terminal to the last full repeat of the TALE DNA binding domain.

In one embodiment, a megaTAL contemplated herein comprises a CTD polypeptide of at least
 5 about amino acids -20 to -1 of a *Xanthomonas* TALE protein (-20 is amino acid 1 of a half-repeat unit C-terminal to the last C-terminal full repeat unit). In particular embodiments, the CTD polypeptide comprises at least about 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids C-terminal to the last full repeat of the TALE DNA binding domain of a *Xanthomonas* TALE protein. In one embodiment, a megaTAL contemplated herein
 10 comprises a CTD polypeptide of at least about amino acids -20 to -1 of a *Ralstonia* TALE protein (-20 is amino acid 1 of a half-repeat unit C-terminal to the last C-terminal full repeat unit). In particular embodiments, the CTD polypeptide comprises at least about 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids C-terminal to the last full repeat of the TALE DNA binding domain of a *Ralstonia* TALE protein.

15 In particular embodiments, a megaTAL contemplated herein, comprises a fusion polypeptide comprising a TALE DNA binding domain engineered to bind a target sequence, a meganuclease engineered to bind and cleave a target sequence, and optionally an NTD and/or CTD polypeptide, optionally joined to each other with one or more linker polypeptides contemplated elsewhere herein. Without wishing to be bound by any particular theory, it is
 20 contemplated that a megaTAL comprising TALE DNA binding domain, and optionally an NTD and/or CTD polypeptide is fused to a linker polypeptide which is further fused to an engineered meganuclease. Thus, the TALE DNA binding domain binds a DNA target sequence that is within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 nucleotides away from the target sequence bound by the DNA binding domain of the meganuclease. In this way,
 25 the megaTALs contemplated herein, increase the specificity and efficiency of genome editing.

In particular embodiments, a megaTAL contemplated herein, comprises one or more TALE DNA binding repeat units and an engineered LHE selected from the group consisting of: I-AabMI, I-AaeMI, I-AniI, I-ApaMI, I-CapIII, I-CapIV, I-CkaMI, I-CpaMI, I-CpaMII, I-CpaMIII, I-CpaMIV, I-CpaMV, I-CpaV, I-CraMI, I-EjeMI, I-GpeMI, I-GpiI, I-GzeMI, I-
 30 GzeMII, I-GzeMIII, I-HjeMI, I-LtrII, I-LtrI, I-LtrWI, I-MpeMI, I-MveMI, I-NcrII, I-Ncrl, I-

NcrMI, I-OheMI, I-OnuI, I-OsoMI, I-OsoMII, I-OsoMIII, I-OsoMIV, I-PanMI, I-PanMII, I-PanMIII, I-PnoMI, I-ScuMI, I-SmaMI, I-SscMI, and I-Vdi141I, or preferably I-CpaMI, I-HjeMI, I-OnuI, I-PanMI, and SmaMI, or more preferably I-OnuI.

In particular embodiments, a megaTAL contemplated herein, comprises an NTD, one
 5 or more TALE DNA binding repeat units, a CTD, and an engineered LHE selected from the group consisting of: I-AabMI, I-AaeMI, I-AniI, I-ApaMI, I-CapIII, I-CapIV, I-CkaMI, I-CpaMI, I-CpaMII, I-CpaMIII, I-CpaMIV, I-CpaMV, I-CpaV, I-CraMI, I-EjeMI, I-GpeMI, I-GpiI, I-GzeMI, I-GzeMII, I-GzeMIII, I-HjeMI, I-LtrII, I-LtrI, I-LtrWI, I-MpeMI, I-MveMI, I-NcrII, I-NcrI, I-NcrMI, I-OheMI, I-OnuI, I-OsoMI, I-OsoMII, I-OsoMIII, I-OsoMIV, I-PanMI,
 10 I-PanMII, I-PanMIII, I-PnoMI, I-ScuMI, I-SmaMI, I-SscMI, and I-Vdi141I, or preferably I-CpaMI, I-HjeMI, I-OnuI, I-PanMI, and SmaMI, or more preferably I-OnuI.

In particular embodiments, a megaTAL contemplated herein, comprises an NTD, about 9.5 to about 11.5 TALE DNA binding repeat units, and an engineered I-OnuI LHE selected from the group consisting of: I-AabMI, I-AaeMI, I-AniI, I-ApaMI, I-CapIII, I-CapIV, I-CkaMI, I-CpaMI, I-CpaMII, I-CpaMIII, I-CpaMIV, I-CpaMV, I-CpaV, I-CraMI, I-EjeMI, I-GpeMI, I-GpiI, I-GzeMI, I-GzeMII, I-GzeMIII, I-HjeMI, I-LtrII, I-LtrI, I-LtrWI, I-MpeMI, I-MveMI, I-NcrII, I-NcrI, I-NcrMI, I-OheMI, I-OnuI, I-OsoMI, I-OsoMII, I-OsoMIII, I-OsoMIV, I-PanMI, I-PanMII, I-PanMIII, I-PnoMI, I-ScuMI, I-SmaMI, I-SscMI, and I-Vdi141I, or preferably I-CpaMI, I-HjeMI, I-OnuI, I-PanMI, and SmaMI, or more preferably I-OnuI.
 20 OnuI.

In particular embodiments, a megaTAL contemplated herein, comprises an NTD of about 122 amino acids to 137 amino acids, about 9.5, about 10.5, or about 11.5 binding repeat units, a CTD of about 20 amino acids to about 85 amino acids, and an engineered I-OnuI LHE selected from the group consisting of: I-AabMI, I-AaeMI, I-AniI, I-ApaMI, I-CapIII, I-CapIV, I-CkaMI, I-CpaMI, I-CpaMII, I-CpaMIII, I-CpaMIV, I-CpaMV, I-CpaV, I-CraMI, I-EjeMI, I-GpeMI, I-GpiI, I-GzeMI, I-GzeMII, I-GzeMIII, I-HjeMI, I-LtrII, I-LtrI, I-LtrWI, I-MpeMI, I-MveMI, I-NcrII, I-NcrI, I-NcrMI, I-OheMI, I-OnuI, I-OsoMI, I-OsoMII, I-OsoMIII, I-OsoMIV, I-PanMI, I-PanMII, I-PanMIII, I-PnoMI, I-ScuMI, I-SmaMI, I-SscMI, and I-Vdi141I, or preferably I-CpaMI, I-HjeMI, I-OnuI, I-PanMI, and SmaMI, or more preferably I-OnuI.
 25 OnuI.
 30 OnuI.

In particular embodiments, a TALEN that binds to and cleaves a target region of a TCR α locus is contemplated. A “TALEN” refers to an engineered nuclease comprising an engineered TALE DNA binding domain contemplated elsewhere herein and an endonuclease domain (or endonuclease half-domain thereof), and optionally comprises one or more linkers and/or additional functional domains, *e.g.*, an end-processing enzymatic domain of an end-processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity. In particular embodiments, a TALEN can be introduced into a T cell with an end-processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity. The TALEN and 3' processing enzyme may be introduced separately, *e.g.*, in different vectors or separate mRNAs, or together, *e.g.*, as a fusion protein, or in a polycistronic construct separated by a viral self-cleaving peptide or an IRES element.

In one embodiment, targeted double-stranded cleavage is achieved with two TALENs, each comprising an endonuclease half-domain that can be used to reconstitute a catalytically active cleavage domain. In another embodiment, targeted double-stranded cleavage is achieved using a single polypeptide comprising a TALE DNA binding domain and two endonuclease half-domains.

TALENs contemplated in particular embodiments comprise an NTD, a TALE DNA binding domain comprising about 3 to 30 repeat units, *e.g.*, about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 repeat units, and an endonuclease domain or half-domain.

TALENs contemplated in particular embodiments comprise an NTD, a TALE DNA binding domain comprising about 3.5 to 30.5 repeat units, *e.g.*, about 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5, 19.5, 20.5, 21.5, 22.5, 23.5, 24.5, 25.5, 26.5, 27.5, 28.5, 29.5, or 30.5 repeat units, a CTD, and an endonuclease domain or half-domain.

TALENs contemplated in particular embodiments comprise an NTD of about 121 amino acids to about 137 amino acids as disclosed elsewhere herein, a TALE DNA binding domain comprising about 9.5 to about 11.5 repeat units (*i.e.*, about 9.5, about 10.5, or about

11.5 repeat units), a CTD of about 20 amino acids to about 85 amino acids, and an endonuclease domain or half domain.

In particular embodiments, a TALEN comprises an endonuclease domain of a type restriction endonuclease. Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.*, Type-IIS) cleave DNA at sites removed from the recognition site and have separable binding and endonuclease domains. In one embodiment, TALENs comprise the endonuclease domain (or endonuclease half-domain) from at least one Type-IIS restriction enzyme and one or more TALE DNA-binding domains contemplated elsewhere herein.

Illustrative examples of Type-IIS restriction endonuclease domains suitable for use in TALENs contemplated in particular embodiments include endonuclease domains of the at least 1633 Type-IIS restriction endonucleases disclosed at “rebase.neb.com/cgi-bin/sublist?S.”

Additional illustrative examples of Type-IIS restriction endonuclease domains suitable for use in TALENs contemplated in particular embodiments include those of endonucleases selected from the group consisting of: Aar I, Ace III, Aci I, Alo I, Alw26 I, Bae I, Bbr7 I, Bbv I, Bbv II, BbvC I, Bcc I, Bce83 I, BceA I, Bcef I, Bcg I, BciV I, Bfi I, Bin I, Bmg I, Bpu10 I, BsaX I, Bsb I, BscA I, BscG I, BseR I, BseY I, Bsi I, Bsm I, BsmA I, BsmF I, Bsp24 I, BspG I, BspM I, BspNC I, Bsr I, BsrB I, BsrD I, BstF5 I, Btr I, Bts I, Cdi I, CjeP I, Drd II, EarI, Eci I, Eco31 I, Eco57 I, Eco57M I, Esp3 I, Fau I, Fin I, Fok I, Gdi II, Gsu I, Hga I, Hin4 II, Hph I, Ksp632 I, Mbo II, Mly I, Mme I, Mnl I, Pfl1108, I Ple I, Ppi I, Psr I, RleA I, Sap I, SfaN I, Sim I, SspD5 I, Sth132 I, Sts I, TspDT I, TspGW I, Tth111 II, UbaP I, Bsa I, and BsmB I.

In one embodiment, a TALEN contemplated herein comprises an endonuclease domain of the Fok I Type-IIS restriction endonuclease.

In one embodiment, a TALEN contemplated herein comprises a TALE DNA binding domain and an endonuclease half-domain from at least one Type-IIS restriction endonuclease to enhance cleavage specificity, optionally wherein the endonuclease half-domain comprises one or more amino acid substitutions or modifications that minimize or prevent homodimerization.

Illustrative examples of cleavage half-domains suitable for use in particular
embodiments contemplated in particular embodiments include those disclosed in U.S. Patent
Publication Nos. 20050064474; 20060188987, 20080131962, 20090311787; 20090305346;
20110014616, and 20110201055, each of which are incorporated by reference herein in its
5 entirety.

In particular embodiments, a zinc finger nuclease (ZFN) that binds to and cleaves a
target site in a TCR α locus is contemplated. A “ZFN” refers to an engineered nuclease
comprising one or more zinc finger DNA binding domains and an endonuclease domain (or
endonuclease half-domain thereof), and optionally comprises one or more linkers and/or
10 additional functional domains, *e.g.*, an end-processing enzymatic domain of an end-processing
enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*,
Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases activity.

In particular embodiments, a ZFN can be introduced into a T cell with an end-
processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease
15 (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerases
activity. The ZFN and 3' processing enzyme may be introduced separately, *e.g.*, in different
vectors or separate mRNAs, or together, *e.g.*, as a fusion protein, or in a polycistronic construct
separated by a viral self-cleaving peptide or an IRES element.

In one embodiment, targeted double-stranded cleavage is achieved using two ZFNs,
20 each comprising an endonuclease half-domain can be used to reconstitute a catalytically active
cleavage domain. In another embodiment, targeted double-stranded cleavage is achieved with
a single polypeptide comprising one or more zinc finger DNA binding domains and two
endonuclease half-domains.

In one embodiment, a ZNF comprises a TALE DNA binding domain contemplated
25 elsewhere herein, a zinc finger DNA binding domain, and an endonuclease domain (or
endonuclease half-domain) contemplated elsewhere herein.

In one embodiment, a ZNF comprises a zinc finger DNA binding domain, and a
meganuclease contemplated elsewhere herein.

In particular embodiments, the ZFN comprises a zinc finger DNA binding domain
30 that has one, two, three, four, five, six, seven, or eight or more zinc finger motifs and an

endonuclease domain (or endonuclease half-domain). Typically, a single zinc finger motif is about 30 amino acids in length. Zinc fingers motifs include both canonical C₂H₂ zinc fingers, and non-canonical zinc fingers such as, for example, C₃H zinc fingers and C₄ zinc fingers.

Zinc finger binding domains can be engineered to bind any DNA sequence. Candidate
 5 zinc finger DNA binding domains for a given 3 bp DNA target sequence have been identified and modular assembly strategies have been devised for linking a plurality of the domains into a multi-finger peptide targeted to the corresponding composite DNA target sequence. Other suitable methods known in the art can also be used to design and construct nucleic acids encoding zinc finger DNA binding domains, *e.g.*, phage display, random mutagenesis,
 10 combinatorial libraries, computer/rational design, affinity selection, PCR, cloning from cDNA or genomic libraries, synthetic construction and the like. (*See, e.g.*, U.S. Pat. No. 5,786,538; Wu *et al.*, *PNAS* 92:344-348 (1995); Jamieson *et al.*, *Biochemistry* 33:5689-5695 (1994); Rebar & Pabo, *Science* 263:671-673 (1994); Choo & Klug, *PNAS* 91:11163-11167 (1994); Choo & Klug, *PNAS* 91: 11168-11172 (1994); Desjarlais & Berg, *PNAS* 90:2256-2260 (1993);
 15 Desjarlais & Berg, *PNAS* 89:7345-7349 (1992); Pomerantz *et al.*, *Science* 267:93-96 (1995); Pomerantz *et al.*, *PNAS* 92:9752-9756 (1995); Liu *et al.*, *PNAS* 94:5525-5530 (1997); Griesman & Pabo, *Science* 275:657-661 (1997); Desjarlais & Berg, *PNAS* 91:11-99-11103 (1994)).

Individual zinc finger motifs bind to a three or four nucleotide sequence. The length of
 20 a sequence to which a zinc finger binding domain is engineered to bind (*e.g.*, a target sequence) will determine the number of zinc finger motifs in an engineered zinc finger binding domain. For example, for ZFNs in which the zinc finger motifs do not bind to overlapping subsites, a six-nucleotide target sequence is bound by a two-finger binding domain; a nine-nucleotide target sequence is bound by a three-finger binding domain, *etc.* In particular embodiments,
 25 DNA binding sites for individual zinc fingers motifs in a target site need not be contiguous, but can be separated by one or several nucleotides, depending on the length and nature of the linker sequences between the zinc finger motifs in a multi-finger binding domain.

In particular embodiments, ZNFs contemplated herein comprise, a zinc finger DNA binding domain comprising two, three, four, five, six, seven or eight or more zinc finger motifs,

and an endonuclease domain or half-domain from at least one Type-IIIS restriction enzyme and one or more TALE DNA-binding domains contemplated elsewhere herein.

In particular embodiments, ZNFs contemplated herein comprise, a zinc finger DNA binding domain comprising three, four, five, six, seven or eight or more zinc finger motifs, and
 5 an endonuclease domain or half-domain from at least one Type-IIIS restriction enzyme selected from the group consisting of: Aar I, Ace III, Aci I, Alo I, Alw26 I, Bae I, Bbr7 I, Bbv I, Bbv II, BbvC I, Bcc I, Bce83 I, BceA I, Bcef I, Bcg I, BciV I, Bfi I, Bin I, Bmg I, Bpu10 I, BsaX I, Bsb I, BscA I, BscG I, BseR I, BseY I, Bsi I, Bsm I, BsmA I, BsmF I, Bsp24 I, BspG I, BspM I, BspNC I, Bsr I, BsrB I, BsrD I, BstF5 I, Btr I, Bts I, Cdi I, CjeP I, Drd II, EarI, Eci I, Eco31
 10 I, Eco57 I, Eco57M I, Esp3 I, Fau I, Fin I, Fok I, Gdi II, Gsu I, Hga I, Hin4 II, Hph I, Ksp632 I, Mbo II, Mly I, Mme I, Mnl I, Pfl1108, I Ple I, Ppi I, Psr I, RleA I, Sap I, SfaN I, Sim I, SspD5 I, Sth132 I, Sts I, TspDT I, TspGW I, Tth111 II, UbaP I, Bsa I, and BsmB I.

In particular embodiments, ZNFs contemplated herein comprise, a zinc finger DNA binding domain comprising three, four, five, six, seven or eight or more zinc finger motifs, and
 15 an endonuclease domain or half-domain from the Fok I Type-IIIS restriction endonuclease.

In one embodiment, a ZFN contemplated herein comprises a zinc finger DNA binding domain and an endonuclease half-domain from at least one Type-IIIS restriction endonuclease to enhance cleavage specificity, optionally wherein the endonuclease half-domain comprises one or more amino acid substitutions or modifications that minimize or prevent
 20 homodimerization.

In various embodiments, a CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR Associated) nuclease system is engineered to bind to, and to introduce single-stranded nicks or double-strand breaks (DSBs) in, one or more TCR α loci. The CRISPR/Cas nuclease system is a recently engineered nuclease system based on a
 25 bacterial system that can be used for mammalian genome engineering. *See, e.g., Jinek et al. (2012) Science 337:816-821; Cong et al. (2013) Science 339:819-823; Mali et al. (2013) Science 339:823-826; Qi et al. (2013) Cell 152:1173-1183; Jinek et al. (2013), eLife 2:e00471; David Segal (2013) eLife 2:e00563; Ran et al. (2013) Nature Protocols 8(11):2281-2308; Zetsche et al. (2015) Cell 163(3):759-771, each of which is incorporated herein by reference in*
 30 its entirety.

In one embodiment, the CRISPR/Cas nuclease system comprises Cas nuclease and one or more RNAs that recruit the Cas nuclease to the target site, *e.g.*, a transactivating crRNA (tracrRNA) and a CRISPR RNA (crRNA), or a single guide RNA (sgRNA). crRNA and tracrRNA can be engineered into one polynucleotide sequence referred to herein as a “single
5 guide RNA” or “sgRNA.”

In one embodiment, the Cas nuclease is engineered as a double-stranded DNA endonuclease or a nickase or catalytically dead Cas, and forms a target complex with a crRNA and a tracrRNA, or sgRNA, for site specific DNA recognition and site-specific cleavage of the protospacer target sequence located within the TCR α locus. The protospacer motif abuts a
10 short protospacer adjacent motif (PAM), which plays a role in recruiting a Cas/RNA complex. Cas polypeptides recognize PAM motifs specific to the Cas polypeptide. Accordingly, the CRISPR/Cas system can be used to target and cleave either or both strands of a double-stranded polynucleotide sequence flanked by particular 3' PAM sequences specific to a particular Cas polypeptide. PAMs may be identified using bioinformatics or using
15 experimental approaches. Esvelt *et al.*, 2013, *Nature Methods*. 10(11):1116-1121, which is hereby incorporated by reference in its entirety.

In one embodiment, the Cas nuclease comprises one or more heterologous DNA binding domains, *e.g.*, a TALE DNA binding domain or zinc finger DNA binding domain. Fusion of the Cas nuclease to TALE or zinc finger DNA binding domains increases the DNA
20 cleavage efficiency and specificity. In a particular embodiment, a Cas nuclease optionally comprises one or more linkers and/or additional functional domains, *e.g.*, an end-processing enzymatic domain of an end-processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerase activity. In particular embodiments, a Cas nuclease can be
25 introduced into a T cell with an end-processing enzyme that exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5' exonuclease (*e.g.*, Trex2), 5' flap endonuclease, helicase or template-independent DNA polymerase activity. The Cas nuclease and 3' processing enzyme may be introduced separately, *e.g.*, in different vectors or separate mRNAs, or together, *e.g.*, as a fusion protein, or in a polycistronic construct separated by a viral self-cleaving peptide or an IRES
30 element.

In various embodiments, the Cas nuclease is Cas9 or Cpf1.

Illustrative examples of Cas9 polypeptides suitable for use in particular embodiments contemplated in particular embodiments may be obtained from bacterial species including, but not limited to: *Enterococcus faecium*, *Enterococcus italicus*, *Listeria innocua*, *Listeria*
 5 *monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, *Streptococcus agalactiae*, *Streptococcus*
anginosus, *Streptococcus bovis*, *Streptococcus dysgalactiae*, *Streptococcus equinus*,
Streptococcus gallolyticus, *Streptococcus macacae*, *Streptococcus mutans*, *Streptococcus*
pseudoporcinus, *Streptococcus pyogenes*, *Streptococcus thermophilus*, *Streptococcus gordonii*,
Streptococcus infantarius, *Streptococcus macedonicus*, *Streptococcus mitis*, *Streptococcus*
 10 *pasteurianus*, *Streptococcus suis*, *Streptococcus vestibularis*, *Streptococcus sanguinis*,
Streptococcus downei, *Neisseria bacilliformis*, *Neisseria cinerea*, *Neisseria flavescens*,
Neisseria lactamica, *Neisseria meningitidis*, *Neisseria subflava*, *Lactobacillus brevis*,
Lactobacillus buchneri, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus*
fermentum, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*,
 15 *Lactobacillus rhamnosus*, *Lactobacillus ruminis*, *Lactobacillus salivarius*, *Lactobacillus*
sanfranciscensis, *Corynebacterium accolens*, *Corynebacterium diphtheriae*, *Corynebacterium*
matruchotii, *Campylobacter jejuni*, *Clostridium perfringens*, *Treponema vincentii*, *Treponema*
phagedenis, and *Treponema denticola*.

Illustrative examples of Cpf1 polypeptides suitable for use in particular embodiments
 20 contemplated in particular embodiments may be obtained from bacterial species including, but
 not limited to: *Francisella spp.*, *Acidaminococcus spp.*, *Prevotella spp.*, *Lachnospiraceae spp.*,
 among others.

Conserved regions of Cas9 orthologs include a central HNH endonuclease domain and
 a split RuvC/RNase H domain. Cpf1 orthologs possess a RuvC/RNase H domain but no
 25 discernable HNH domain. The HNH and RuvC-like domains are each responsible for cleaving
 one strand of the double-stranded DNA target sequence. The HNH domain of the Cas9
 nuclease polypeptide cleaves the DNA strand complementary to the tracrRNA:crRNA or
 sgRNA. The RuvC-like domain of the Cas9 nuclease cleaves the DNA strand that is not-
 complementary to the tracrRNA:crRNA or sgRNA. Cpf1 is predicted to act as a dimer
 30 wherein each RuvC-like domain of Cpf1 cleaves either the complementary or non-

complementary strand of the target site. In particular embodiments, a Cas9 nuclease variant (e.g., Cas9 nickase) is contemplated comprising one or more amino acids additions, deletions, mutations, or substitutions in the HNH or RuvC-like endonuclease domains that decreases or eliminates the nuclease activity of the variant domain.

5 Illustrative examples of Cas9 HNH mutations that decrease or eliminate the nuclease activity in the domain include, but are not limited to: *S. pyogenes* (D10A); *S. thermophilis* (D9A); *T. denticola* (D13A); and *N. meningitidis* (D16A).

10 Illustrative examples of Cas9 RuvC-like domain mutations that decrease or eliminate the nuclease activity in the domain include, but are not limited to: *S. pyogenes* (D839A, H840A, or N863A); *S. thermophilis* (D598A, H599A, or N622A); *T. denticola* (D878A, H879A, or N902A); and *N. meningitidis* (D587A, H588A, or N611A).

E. CHIMERIC ANTIGEN RECEPTORS (CARs)

In particular embodiments, T cells comprising one or more modified TCR α alleles are engineered to express one or more chimeric antigen receptors (CARs).

15 In one embodiment, T cells are engineered by introducing a DSB in one or more TCR α alleles and by subsequently transducing the T cells with a vector encoding a CAR.

In one embodiment, T cells are engineered by introducing a DSB in one or more TCR α alleles in the presence of a donor repair template encoding a CAR. In a particular embodiment, a CAR is inserted at a DSB in a single TCR α allele.

20 In one embodiment, the engineered T cells contemplated herein comprise a CAR that is not inserted at a TCR α allele.

In various embodiments, the genome edited T cells express CARs that redirect cytotoxicity toward tumor cells. CARs are molecules that combine antibody-based specificity for a target antigen (e.g., tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-tumor cellular immune activity. As used herein, the term, “chimeric,” describes being composed of parts of different proteins or DNAs from different origins.

In various embodiments, a CAR comprises an extracellular domain that binds to a specific target antigen (also referred to as a binding domain or antigen-specific binding

domain), a transmembrane domain and an intracellular signaling domain. The main characteristic of CARs are their ability to redirect immune effector cell specificity, thereby triggering proliferation, cytokine production, phagocytosis or production of molecules that can mediate cell death of the target antigen expressing cell in a major histocompatibility
 5 (MHC) independent manner, exploiting the cell specific targeting abilities of monoclonal antibodies, soluble ligands or cell specific coreceptors.

In particular embodiments, CARs comprise an extracellular binding domain that specifically binds to a target polypeptide, *e.g.*, a target antigen, expressed on tumor cell. As used herein, the terms, “binding domain,” “extracellular domain,” “extracellular binding
 10 domain,” “antigen binding domain,” “antigen-specific binding domain,” and “extracellular antigen specific binding domain,” are used interchangeably and provide a CAR with the ability to specifically bind to the target antigen of interest. A binding domain may comprise any protein, polypeptide, oligopeptide, or peptide that possesses the ability to specifically recognize and bind to a biological molecule (*e.g.*, a cell surface receptor or tumor protein, lipid,
 15 polysaccharide, or other cell surface target molecule, or component thereof). A binding domain includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced binding partner for a biological molecule of interest.

In particular embodiments, the extracellular binding domain comprises an antibody or antigen binding fragment thereof.

20 An “antibody” refers to a binding agent that is a polypeptide comprising at least a light chain or heavy chain immunoglobulin variable region which specifically recognizes and binds an epitope of a target antigen, such as a peptide, lipid, polysaccharide, or nucleic acid containing an antigenic determinant, such as those recognized by an immune cell. Antibodies include antigen binding fragments, *e.g.*, Camel Ig (a camelid antibody or VHH fragment
 25 thereof), Ig NAR, Fab fragments, Fab' fragments, F(ab)'2 fragments, F(ab)'3 fragments, Fv, single chain Fv antibody (“scFv”), bis-scFv, (scFv)₂, minibody, diabody, triabody, tetrabody, disulfide stabilized Fv protein (“dsFv”), and single-domain antibody (sdAb, Nanobody) or other antibody fragments thereof. The term also includes genetically engineered forms such as chimeric antibodies (for example, humanized murine antibodies), heteroconjugate antibodies
 30 (such as, bispecific antibodies) and antigen binding fragments thereof. *See also*, Pierce Catalog

and Handbook, 1994-1995 (Pierce Chemical Co., Rockford, IL); Kuby, J., Immunology, 3rd Ed., W. H. Freeman & Co., New York, 1997.

In one preferred embodiment, the binding domain is an scFv.

In another preferred embodiment, the binding domain is a camelid antibody.

5 In particular embodiments, the CAR comprises an extracellular domain that binds an antigen selected from the group consisting of: alpha folate receptor, 5T4, $\alpha_v\beta_6$ integrin, BCMA, B7-H3, B7-H6, CAIX, CD16, CD19, CD20, CD22, CD30, CD33, CD44, CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD138, CD171, CEA, CSPG4, EGFR, EGFR family including ErbB2 (HER2), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FR α , GD2, GD3, Glypican-3 (GPC3), HLA-A1+MAGE1, HLA-A2+MAGE1, HLA-A3+MAGE1, HLA-A1+NY-ESO-1, HLA-A2+NY-ESO-1, HLA-A3+NY-ESO-1, IL-11R α , IL-13R α 2, Lambda, Lewis-Y, Kappa, Mesothelin, Muc1, Muc16, NCAM, NKG2D Ligands, NY-ESO-1, PRAME, PSCA, PSMA, ROR1, SSX, Survivin, TAG72, TEMs, VEGFR2, and WT-1.

15 In particular embodiments, the CAR comprises an extracellular domain that binds an antigen selected from the group consisting of: BCMA, CD19, CSPG4, PSCA, ROR1, and TAG72.

In particular embodiments, the CARs comprise an extracellular binding domain, *e.g.*, antibody or antigen binding fragment thereof that binds an antigen, wherein the antigen is an MHC-peptide complex, such as a class I MHC-peptide complex or a class II MHC-peptide complex.

In certain embodiments, the CARs comprise linker residues between the various domains. A “variable region linking sequence,” is an amino acid sequence that connects a heavy chain variable region to a light chain variable region and provides a spacer function compatible with interaction of the two sub-binding domains so that the resulting polypeptide retains a specific binding affinity to the same target molecule as an antibody that comprises the same light and heavy chain variable regions. In particular embodiments, a linker separates one or more heavy or light chain variable domains, hinge domains, transmembrane domains, co-stimulatory domains, and/or primary signaling domains. In particular embodiments, CARs comprise one, two, three, four, or five or more linkers. In

particular embodiments, the length of a linker is about 1 to about 25 amino acids, about 5 to about 20 amino acids, or about 10 to about 20 amino acids, or any intervening length of amino acids. In some embodiments, the linker is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more amino acids long.

5 In particular embodiments, the binding domain of the CAR is followed by one or more “spacer domains,” which refers to the region that moves the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation (Patel *et al.*, *Gene Therapy*, 1999; 6: 412-419). The spacer domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. In certain embodiments, a spacer
10 domain is a portion of an immunoglobulin, including, but not limited to, one or more heavy chain constant regions, *e.g.*, CH2 and CH3. The spacer domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

In one embodiment, the spacer domain comprises the CH2 and CH3 of IgG1, IgG4, or
15 IgD.

In one embodiment, the binding domain of the CAR is linked to one or more “hinge domains,” which plays a role in positioning the antigen binding domain away from the effector cell surface to enable proper cell/cell contact, antigen binding and activation. A CAR generally comprises one or more hinge domains between the binding domain and the transmembrane
20 domain (TM). The hinge domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source. The hinge domain can include the amino acid sequence of a naturally occurring immunoglobulin hinge region or an altered immunoglobulin hinge region.

Illustrative hinge domains suitable for use in the CARs described herein include the hinge region derived from the extracellular regions of type 1 membrane proteins such as CD8 α ,
25 and CD4, which may be wild-type hinge regions from these molecules or may be altered. In another embodiment, the hinge domain comprises a CD8 α hinge region.

In one embodiment, the hinge is a PD-1 hinge or CD152 hinge.

The “transmembrane domain” is the portion of the CAR that fuses the extracellular binding portion and intracellular signaling domain and anchors the CAR to the

plasma membrane of the immune effector cell. The TM domain may be derived either from a natural, synthetic, semi-synthetic, or recombinant source.

Illustrative TM domains may be derived from (*i.e.*, comprise at least the transmembrane region(s) of the alpha or beta chain of the T-cell receptor, CD3 δ , CD3 ϵ ,
 5 CD3 γ , CD3 ζ , CD4, CD5, CD8 α , CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD154, and PD-1.

In one embodiment, a CAR comprises a TM domain derived from CD8 α . In another embodiment, a CAR contemplated herein comprises a TM domain derived from CD8 α and a short oligo- or polypeptide linker, preferably between 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino
 10 acids in length that links the TM domain and the intracellular signaling domain of the CAR. A glycine-serine linker provides a particularly suitable linker.

In particular embodiments, a CAR comprises an intracellular signaling domain. An “intracellular signaling domain,” refers to the part of a CAR that participates in transducing the message of effective CAR binding to a target antigen into the interior of the immune effector
 15 cell to elicit effector cell function, *e.g.*, activation, cytokine production, proliferation and cytotoxic activity, including the release of cytotoxic factors to the CAR-bound target cell, or other cellular responses elicited with antigen binding to the extracellular CAR domain.

Thus, the term “intracellular signaling domain” refers to the portion of a protein which transduces the effector function signal and that directs the cell to perform a specialized
 20 function. While usually the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire domain. To the extent that a truncated portion of an intracellular signaling domain is used, such truncated portion may be used in place of the entire domain as long as it transduces the effector function signal. The term intracellular signaling domain is meant to include any truncated portion of the intracellular signaling domain
 25 sufficient to transducing effector function signal.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary or costimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of intracellular signaling domains: primary signaling domains that initiate antigen-dependent primary activation through
 30 the TCR (*e.g.*, a TCR/CD3 complex) and costimulatory signaling domains that act in an

antigen-independent manner to provide a secondary or costimulatory signal. In preferred embodiments, a CAR comprises an intracellular signaling domain that comprises one or more “costimulatory signaling domains” and a “primary signaling domain.”

Primary signaling domains regulate primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

Illustrative examples of ITAM containing primary signaling domains suitable for use in CARs contemplated in particular embodiments include those derived from FcR γ , FcR β , CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , CD22, CD79a, CD79b, and CD66d. In particular preferred embodiments, a CAR comprises a CD3 ζ primary signaling domain and one or more costimulatory signaling domains. The intracellular primary signaling and costimulatory signaling domains may be linked in any order in tandem to the carboxyl terminus of the transmembrane domain.

In particular embodiments, a CAR comprises one or more costimulatory signaling domains to enhance the efficacy and expansion of T cells expressing CAR receptors. As used herein, the term, “costimulatory signaling domain,” or “costimulatory domain,” refers to an intracellular signaling domain of a costimulatory molecule.

Illustrative examples of such costimulatory molecules suitable for use in CARs contemplated in particular embodiments include TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DAP10, LAT, NKD2C, SLP76, TRIM, and ZAP70. In one embodiment, a CAR comprises one or more costimulatory signaling domains selected from the group consisting of CD28, CD137, and CD134, and a CD3 ζ primary signaling domain.

In various embodiments, the CAR comprises: an extracellular domain that binds an antigen selected from the group consisting of: BCMA, CD19, CSPG4, PSCA, ROR1, and TAG72; a transmembrane domain isolated from a polypeptide selected from the group consisting of: CD4, CD8 α , CD154, and PD-1; one or more intracellular costimulatory signaling domains isolated from a polypeptide selected from the group consisting of: CD28, CD134, and CD137; and a signaling domain isolated from a polypeptide selected from the

group consisting of: FcR γ , FcR β , CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , CD22, CD79a, CD79b, and CD66d.

F. POLYPEPTIDES

Various polypeptides are contemplated herein, including, but not limited to, CARs, 5 meganucleases, megaTALs, TALENs, ZFNs, and Cas nucleases, fusion polypeptides, and vectors that express polypeptides.

“Polypeptide,” “polypeptide fragment,” “peptide” and “protein” are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.*, as a sequence of amino acids. In one embodiment, a “polypeptide” includes fusion 10 polypeptides and other variants. Polypeptides can be prepared using any of a variety of well-known recombinant and/or synthetic techniques. Polypeptides are not limited to a specific length, *e.g.*, they may comprise a full length protein sequence, a fragment of a full length protein, or a fusion protein, and may include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as 15 other modifications known in the art, both naturally occurring and non-naturally occurring.

An “isolated peptide” or an “isolated polypeptide” and the like, as used herein, refer to *in vitro* isolation and/or purification of a peptide or polypeptide molecule from a cellular environment, and from association with other components of the cell, *i.e.*, it is not significantly associated with *in vivo* substances.

20 Illustrative examples of polypeptides contemplated in particular embodiments include, but are not limited to meganucleases, megaTALs, TALENs, ZFNs, Cas nucleases, end-processing nucleases, therapeutic polypeptides and fusion polypeptides and variants thereof.

Polypeptide variants include biologically active “polypeptide fragments.” As used herein, the term “biologically active fragment” or “minimal biologically active fragment” refers 25 to a polypeptide fragment that retains at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the naturally occurring polypeptide activity. Polypeptide fragments refer to a polypeptide, which can be monomeric or multimeric that has an amino-terminal deletion, a carboxyl-terminal deletion, and/or an internal deletion or substitution of one or more amino acids of a

naturally-occurring or recombinantly-produced polypeptide. In certain embodiments, a polypeptide fragment can comprise an amino acid chain at least 5 to about 1700 amino acids long. It will be appreciated that in certain embodiments, fragments are at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700 or more amino acids long.

Illustrative examples of polypeptide fragments include DNA binding domains, nuclease domains, antibody fragments, extracellular ligand binding domains, signaling domains, transmembrane domains, multimerization domains, and the like.

Polypeptides include “polypeptide variants.” Polypeptide variants may differ from a naturally occurring polypeptide in one or more amino acid substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more amino acids of the above polypeptide sequences. For example, in particular embodiments, it may be desirable to improve the biological properties of a polypeptide by introducing one or more substitutions, deletions, additions and/or insertions into the polypeptide. In particular embodiments, polypeptides include polypeptides having at least about 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% amino acid identity to any of the reference sequences contemplated herein, typically where the variant maintains at least one biological activity of the reference sequence.

As noted above, polypeptides may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of a reference polypeptide can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985, *Proc. Natl. Acad. Sci. USA*. 82: 488-492), Kunkel *et al.*, (1987, *Methods in Enzymol*, 154: 367-382), U.S. Pat. No. 4,873,192, Watson, J. D. *et al.*, (*Molecular Biology of the Gene*, Fourth Edition, Benjamin/Cummings, Menlo Park, Calif., 1987) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the

protein of interest may be found in the model of Dayhoff *et al.*, (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.).

In certain embodiments, a variant will contain one or more conservative substitutions. A “conservative substitution” is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydrophobic nature of the polypeptide to be substantially unchanged. Modifications may be made in the structure of the polynucleotides and polypeptides contemplated in particular embodiments, polypeptides include polypeptides having at least about and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, variant polypeptide, one skilled in the art, for example, can change one or more of the codons of the encoding DNA sequence, *e.g.*, according to Table 1.

TABLE 1- Amino Acid Codons

Amino Acids	One letter code	Three letter code	Codons					
Alanine	A	Ala	GCA	GCC	GCG	GCU		
Cysteine	C	Cys	UGC	UGU				
Aspartic acid	D	Asp	GAC	GAU				
Glutamic acid	E	Glu	GAA	GAG				
Phenylalanine	F	Phe	UUC	UUU				
Glycine	G	Gly	GGA	GGC	GGG	GGU		
Histidine	H	His	CAC	CAU				
Isoleucine	I	Iso	AUA	AUC	AUU			
Lysine	K	Lys	AAA	AAG				
Leucine	L	Leu	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	M	Met	AUG					
Asparagine	N	Asn	AAC	AAU				
Proline	P	Pro	CCA	CCC	CCG	CCU		

Glutamine	Q	Gln	CAA	CAG				
Arginine	R	Arg	AGA	AGG	CGA	CGC	CGG	CGU
Serine	S	Ser	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	T	Thr	ACA	ACC	ACG	ACU		
Valine	V	Val	GUA	GUC	GUG	GUU		
Tryptophan	W	Trp	UGG					
Tyrosine	Y	Tyr	UAC	UAU				

Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs well known in the art, such as DNASTAR, DNA Strider, Geneious, Mac Vector, or Vector NTI software. Preferably, amino acid changes in the protein variants disclosed herein are

5 conservative amino acid changes, *i.e.*, substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and

10 uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and generally can be made without altering a biological activity of a resulting molecule. Those of skill in this art recognize that, in general, single

15 amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al. Molecular Biology of the Gene*, 4th Edition, 1987, The Benjamin/Cummings Pub. Co., p.224).

Polypeptide variants further include glycosylated forms, aggregative conjugates with other molecules, and covalent conjugates with unrelated chemical moieties (*e.g.*, pegylated

20 molecules). Covalent variants can be prepared by linking functionalities to groups which are found in the amino acid chain or at the N- or C-terminal residue, as is known in the art. Variants also include allelic variants, species variants, and muteins. Truncations or deletions of regions which do not affect functional activity of the proteins are also variants.

In one embodiment, where expression of two or more polypeptides is desired, the polynucleotide sequences encoding them can be separated by an IRES sequence as disclosed elsewhere herein.

Polypeptides contemplated in particular embodiments include fusion polypeptides. In particular embodiments, fusion polypeptides and polynucleotides encoding fusion polypeptides are provided. Fusion polypeptides and fusion proteins refer to a polypeptide having at least two, three, four, five, six, seven, eight, nine, or ten polypeptide segments.

In another embodiment, two or more polypeptides can be expressed as a fusion protein that comprises one or more self-cleaving polypeptide sequences as disclosed elsewhere herein.

In one embodiment, a fusion protein contemplated herein comprises one or more DNA binding domains and one or more nucleases, and one or more linker and/or self-cleaving polypeptides.

In one embodiment, a fusion protein contemplated herein comprises one or more exodomains, extracellular ligand binding domains, or antigen binding domain, a transmembrane domain, and one or more intracellular signaling domains, and optionally one or more multimerization domains.

Illustrative examples of fusion proteins contemplated in particular embodiments, polypeptides include polypeptides having at least about include, but are not limited to: megaTALs, TALENs, ZFNs, Cas nucleases, end-processing nucleases, CARs, and other polypeptides.

Fusion polypeptides are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. In particular embodiments, the polypeptides of the fusion protein can be in any order. Fusion polypeptides or fusion proteins can also include conservatively modified variants, polymorphic variants, alleles, mutants, subsequences, and interspecies homologs, so long as the desired activity of the fusion polypeptide is preserved. Fusion polypeptides may be produced by chemical synthetic methods or by chemical linkage between the two moieties or may generally be prepared using other standard techniques. Ligated DNA sequences comprising the fusion polypeptide are operably linked to suitable transcriptional or translational control elements as disclosed elsewhere herein.

Fusion polypeptides may optionally comprise a linker that can be used to link the one or more polypeptides or domains within a polypeptide. A peptide linker sequence may be employed to separate any two or more polypeptide components by a distance sufficient to ensure that each polypeptide folds into its appropriate secondary and tertiary structures so as to allow the polypeptide domains to exert their desired functions. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.*, *Gene* 40:39-46, 1985; Murphy *et al.*, *Proc. Natl. Acad. Sci. USA* 83:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. Linker sequences are not required when a particular fusion polypeptide segment contains non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference. Preferred linkers are typically flexible amino acid subsequences which are synthesized as part of a recombinant fusion protein. Linker polypeptides can be between 1 and 200 amino acids in length, between 1 and 100 amino acids in length, or between 1 and 50 amino acids in length, including all integer values in between.

Exemplary linkers include, but are not limited to the following amino acid sequences: glycine polymers (G)_n; glycine-serine polymers (G₁₋₅S₁₋₅)_n, where n is an integer of at least one, two, three, four, or five; glycine-alanine polymers; alanine-serine polymers; GGG (SEQ ID NO: 14); DGGGS (SEQ ID NO: 15); TGEKP (SEQ ID NO: 16) (*see e.g.*, Liu *et al.*, *PNAS* 5525-5530 (1997)); GGRR (SEQ ID NO: 17) (Pomerantz *et al.* 1995, *supra*); (GGGGS)_n (SEQ ID NO: 18) (Kim *et al.*, *PNAS* 93, 1156-1160 (1996.)); EGKSSGSGSESKVD (SEQ ID NO: 19) (Chaudhary *et al.*, 1990, *Proc. Natl. Acad. Sci. U.S.A.* 87:1066-1070); KESGSVSSEQLAQFRSLD (SEQ ID NO: 20) (Bird *et al.*, 1988, *Science* 242:423-426), GGRRGGGS (SEQ ID NO: 21); LRQRDGERP (SEQ ID NO: 22); LRQKDGGGSERP (SEQ

ID NO: 23); LRQKD(GGGS)₂ERP (SEQ ID NO: 24). Alternatively, flexible linkers can be rationally designed using a computer program capable of modeling both DNA-binding sites and the peptides themselves (Desjarlais & Berg, *PNAS* 90:2256-2260 (1993), *PNAS* 91:11099-11103 (1994) or by phage display methods.

5 Fusion polypeptides may further comprise a polypeptide cleavage signal between each of the polypeptide domains described herein or between an endogenous open reading frame and a polypeptide encoded by a donor repair template. In addition, a polypeptide cleavage site can be put into any linker peptide sequence. Exemplary polypeptide cleavage signals include polypeptide cleavage recognition sites such as protease cleavage sites, nuclease cleavage sites
10 (*e.g.*, rare restriction enzyme recognition sites, self-cleaving ribozyme recognition sites), and self-cleaving viral oligopeptides (*see* deFelipe and Ryan, 2004. *Traffic*, 5(8); 616-26).

Suitable protease cleavages sites and self-cleaving peptides are known to the skilled person (*see, e.g.*, in Ryan *et al.*, 1997. *J. Gener. Virol.* 78, 699-722; Scymczak *et al.* (2004) Nature Biotech. 5, 589-594). Exemplary protease cleavage sites include, but are not limited to
15 the cleavage sites of potyvirus NIa proteases (*e.g.*, tobacco etch virus protease), potyvirus HC proteases, potyvirus P1 (P35) proteases, byovirus NIa proteases, byovirus RNA-2-encoded proteases, aphthovirus L proteases, enterovirus 2A proteases, rhinovirus 2A proteases, picorna 3C proteases, comovirus 24K proteases, nepovirus 24K proteases, RTSV (rice tungro spherical virus) 3C-like protease, PYVF (parsnip yellow fleck virus) 3C-like protease, heparin,
20 thrombin, factor Xa and enterokinase. Due to its high cleavage stringency, TEV (tobacco etch virus) protease cleavage sites are preferred in one embodiment, *e.g.*, EXXYXQ(G/S) (SEQ ID NO: 25), for example, ENLYFQG (SEQ ID NO: 26) and ENLYFQS (SEQ ID NO: 27), wherein X represents any amino acid (cleavage by TEV occurs between Q and G or Q and S).

In certain embodiments, the self-cleaving polypeptide site comprises a 2A or 2A-like
25 site, sequence or domain (Donnelly *et al.*, 2001. *J. Gen. Virol.* 82:1027-1041). In a particular embodiment, the viral 2A peptide is an aphthovirus 2A peptide, a potyvirus 2A peptide, or a cardiovirus 2A peptide.

In one embodiment, the viral 2A peptide is selected from the group consisting of: a foot-and-mouth disease virus (FMDV) 2A peptide, an equine rhinitis A virus (ERAV) 2A

peptide, a Thosea asigna virus (TaV) 2A peptide, a porcine teschovirus-1 (PTV-1) 2A peptide, a Theilovirus 2A peptide, and an encephalomyocarditis virus 2A peptide.

Illustrative examples of 2A sites are provided in Table 2.

TABLE 2: Exemplary 2A sites include the following sequences:

SEQ ID NO: 28	GSGATNFSLLKQAGDVEENPGP
SEQ ID NO: 29	ATNFSLLKQAGDVEENPGP
SEQ ID NO: 30	LLKQAGDVEENPGP
SEQ ID NO: 31	GSGEGRGSLLTCGDVEENPGP
SEQ ID NO: 32	EGRGSLLTCGDVEENPGP
SEQ ID NO: 33	LLTCGDVEENPGP
SEQ ID NO: 34	GSGQCTNYALLKLAGDVESNPGP
SEQ ID NO: 35	QCTNYALLKLAGDVESNPGP
SEQ ID NO: 36	LLKLAGDVESNPGP
SEQ ID NO: 37	GSGVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 38	VKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 39	LLKLAGDVESNPGP
SEQ ID NO: 40	LLNFDLLKLAGDVESNPGP
SEQ ID NO: 41	TLNFDLLKLAGDVESNPGP
SEQ ID NO: 42	LLKLAGDVESNPGP
SEQ ID NO: 43	NFDLLKLAGDVESNPGP
SEQ ID NO: 44	QLLNFDLLKLAGDVESNPGP
SEQ ID NO: 45	APVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 46	VTELLYRMKRAETCYCPRLLAIHPTEARHKQKIVAPVKQT
SEQ ID NO: 47	LNFDLLKLAGDVESNPGP
SEQ ID NO: 48	LLAIHPTEARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP
SEQ ID NO: 49	EARHKQKIVAPVKQTLNFDLLKLAGDVESNPGP

G. POLYNUCLEOTIDES

In particular embodiments, polynucleotides encoding a polypeptide or fusion polypeptide contemplated herein are provided. As used herein, the terms “polynucleotide” or “nucleic acid” refer to deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and DNA/RNA hybrids. Polynucleotides may be single-stranded or double-stranded. Polynucleotides include, but are not limited to: pre-messenger RNA (pre-mRNA), messenger RNA (mRNA), RNA, short interfering RNA (siRNA), short hairpin RNA (shRNA), microRNA (miRNA), ribozymes, synthetic RNA, genomic RNA (gRNA), plus strand RNA (RNA(+)), minus strand RNA (RNA(-)), tracrRNA, crRNA, single guide RNA (sgRNA), synthetic RNA, genomic DNA (gDNA), PCR amplified DNA, complementary DNA (cDNA), synthetic DNA, or recombinant DNA.

Polynucleotides refer to a polymeric form of nucleotides of at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 40, at least 50, at least 100, at least 200, at least 300, at least 400, at least 500, at least 1000, at least 5000, at least 10000, or at least 15000 or more nucleotides in length, either ribonucleotides or deoxyribonucleotides or a modified form of either type of nucleotide, as well as all intermediate lengths. It will be readily understood that “intermediate lengths,” in this context, means any length between the quoted values, such as 6, 7, 8, 9, *etc.*, 101, 102, 103, *etc.*; 151, 152, 153, *etc.*; 201, 202, 203, *etc.* In particular embodiments, polynucleotides or variants have at least or about 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a reference sequence.

In various illustrative embodiments, polynucleotides contemplated herein include, but are not limited to polynucleotides encoding meganucleases, megaTALs, TALENs, ZFNs, Cas nucleases, end-processing nucleases, CARs, therapeutic polypeptides, and polynucleotides comprising expression vectors, viral vectors, and transfer plasmids.

As used herein, the terms “polynucleotide variant” and “variant” and the like refer to polynucleotides displaying substantial sequence identity with a reference polynucleotide sequence or polynucleotides that hybridize with a reference sequence under stringent conditions that are defined hereinafter. These terms also encompass polynucleotides that are

distinguished from a reference polynucleotide by the addition, deletion, substitution, or modification of at least one nucleotide. Accordingly, the terms “polynucleotide variant” and “variant” include polynucleotides in which one or more nucleotides have been added or deleted, or modified, or replaced with different nucleotides. In this regard, it is well understood
 5 in the art that certain alterations inclusive of mutations, additions, deletions and substitutions can be made to a reference polynucleotide whereby the altered polynucleotide retains the biological function or activity of the reference polynucleotide.

In one embodiment, a polynucleotide comprises a nucleotide sequence that hybridizes to a target nucleic acid sequence under stringent conditions. To hybridize under “stringent
 10 conditions” describes hybridization protocols in which nucleotide sequences at least 60% identical to each other remain hybridized. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence
 15 hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at T_m , 50% of the probes are occupied at equilibrium.

The recitations “sequence identity” or, for example, comprising a “sequence 50% identical to,” as used herein, refer to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a
 20 “percentage of sequence identity” may be calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, I) or the identical amino acid residue (*e.g.*, Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number
 25 of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Included are nucleotides and polypeptides having at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any of the reference sequences described herein, typically where the polypeptide variant maintains at least
 30 one biological activity of the reference polypeptide.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include “reference sequence,” “comparison window,” “sequence identity,” “percentage of sequence identity,” and “substantial identity”. A “reference sequence” is at least 12 but frequently 15 to 18 and often at least 25 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (*i.e.*, only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a “comparison window” to identify and compare local regions of sequence similarity. A “comparison window” refers to a conceptual segment of at least 6 contiguous positions, usually about 50 to about 100, more usually about 100 to about 150 in which a sequence is compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. The comparison window may comprise additions or deletions (*i.e.*, gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (*i.e.*, resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, *Nucl. Acids Res.* 25:3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons Inc., 1994-1998, Chapter 15.

An “isolated polynucleotide,” as used herein, refers to a polynucleotide that has been purified from the sequences which flank it in a naturally-occurring state, *e.g.*, a DNA fragment that has been removed from the sequences that are normally adjacent to the fragment. In particular embodiments, an “isolated polynucleotide” refers to a complementary DNA (cDNA), a recombinant polynucleotide, a synthetic polynucleotide, or other polynucleotide that does not exist in nature and that has been made by the hand of man.

Terms that describe the orientation of polynucleotides include: 5' (normally the end of the polynucleotide having a free phosphate group) and 3' (normally the end of the polynucleotide having a free hydroxyl (OH) group). Polynucleotide sequences can be annotated in the 5' to 3' orientation or the 3' to 5' orientation. For DNA and mRNA, the 5' to 3' strand is designated the “sense,” “plus,” or “coding” strand because its sequence is identical to the sequence of the pre-messenger (pre-mRNA) [except for uracil (U) in RNA, instead of thymine (T) in DNA]. For DNA and mRNA, the complementary 3' to 5' strand which is the strand transcribed by the RNA polymerase is designated as “template,” “antisense,” “minus,” or “non-coding” strand. As used herein, the term “reverse orientation” refers to a 5' to 3' sequence written in the 3' to 5' orientation or a 3' to 5' sequence written in the 5' to 3' orientation.

The terms “complementary” and “complementarity” refer to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, the complementary strand of the DNA sequence 5' A G T C A T G 3' is 3' T C A G T A C 5'. The latter sequence is often written as the reverse complement with the 5' end on the left and the 3' end on the right, 5' C A T G A C T 3'. A sequence that is equal to its reverse complement is said to be a palindromic sequence. Complementarity can be “partial,” in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there can be “complete” or “total” complementarity between the nucleic acids.

The term “nucleic acid cassette” as used herein refers to genetic sequences within the vector which can express one or more RNAs. In one embodiment, the nucleic acid cassette contains one or more polynucleotide(s)-of-interest. In another embodiment, the nucleic acid cassette contains one or more expression control sequences operably linked to one or more polynucleotide(s)-of-interest. Vectors may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 or more nucleic acid cassettes. The cassette can be removed from or inserted into other polynucleotide sequences, *e.g.*, a plasmid or viral vector, as a single unit.

Polynucleotides include polynucleotide(s)-of-interest. As used herein, the term “polynucleotide-of-interest” refers to a polynucleotide encoding a polypeptide or fusion polypeptide or a polynucleotide that serves as a template for the transcription of an inhibitory polynucleotide, as contemplated herein.

Moreover, it will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that may encode a polypeptide, or fragment of variant thereof, as contemplated herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene.

5 Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated in particular embodiments, for example polynucleotides that are optimized for human and/or primate codon selection. In one embodiment, polynucleotides comprising particular allelic sequences are provided. Alleles are endogenous polynucleotide sequences that are altered as a result of one or more mutations, such as deletions, additions and/or
10 substitutions of nucleotides.

In a certain embodiment, a polynucleotide-of-interest comprises a donor repair template encoding a CAR.

In a certain embodiment, a polynucleotide-of-interest comprises an inhibitory polynucleotide including, but not limited to, a crRNA, a tracrRNA, a single guide RNA
15 (sgRNA), an siRNA, an miRNA, an shRNA, a ribozyme or another inhibitory RNA.

As used herein, the terms “siRNA” or “short interfering RNA” refer to a short polynucleotide sequence that mediates a process of sequence-specific post-transcriptional gene silencing, translational inhibition, transcriptional inhibition, or epigenetic RNAi in animals (Zamore *et al.*, 2000, *Cell*, 101, 25-33; Fire *et al.*, 1998, *Nature*, 391, 806; Hamilton *et al.*,
20 1999, *Science*, 286, 950-951; Lin *et al.*, 1999, *Nature*, 402, 128-129; Sharp, 1999, *Genes & Dev.*, 13, 139-141; and Strauss, 1999, *Science*, 286, 886).

As used herein, the terms “miRNA” or “microRNA” s refer to small non-coding RNAs of 20–22 nucleotides, typically excised from ~70 nucleotide fold-back RNA precursor structures known as pre-miRNAs. miRNAs negatively regulate their targets in one of two
25 ways depending on the degree of complementarity between the miRNA and the target.

As used herein, the terms “shRNA” or “short hairpin RNA” refer to double-stranded structure that is formed by a single self-complementary RNA strand.

As used herein, the term “ribozyme” refers to a catalytically active RNA molecule capable of site-specific cleavage of target mRNA.

In one embodiment, a donor repair template comprises an inhibitory RNA comprises one or more regulatory sequences, such as, for example, a strong constitutive pol III, *e.g.*, human or mouse U6 snRNA promoter, the human and mouse H1 RNA promoter, or the human tRNA-val promoter, or a strong constitutive pol II promoter, as described elsewhere herein.

5 The polynucleotides contemplated in particular embodiments, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters and/or enhancers, untranslated regions (UTRs), Kozak sequences, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, internal ribosomal entry sites (IRES), recombinase recognition sites (*e.g.*, LoxP, FRT, and Att sites), termination codons,
 10 transcriptional termination signals, and polynucleotides encoding self-cleaving polypeptides, epitope tags, as disclosed elsewhere herein or as known in the art, such that their overall length may vary considerably. It is therefore contemplated in particular embodiments that a polynucleotide fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA
 15 protocol.

Polynucleotides can be prepared, manipulated, expressed and/or delivered using any of a variety of well-established techniques known and available in the art. In order to express a desired polypeptide, a nucleotide sequence encoding the polypeptide, can be inserted into appropriate vector.

20 Illustrative examples of vectors include, but are not limited to plasmid, autonomously replicating sequences, and transposable elements, *e.g.*, Sleeping Beauty, PiggyBac.

Additional Illustrative examples of vectors include, without limitation, plasmids, phagemids, cosmids, artificial chromosomes such as yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), or P1-derived artificial chromosome (PAC),
 25 bacteriophages such as lambda phage or M13 phage, and animal viruses.

Illustrative examples of viruses useful as vectors include, without limitation, retrovirus (including lentivirus), adenovirus, adeno-associated virus, herpesvirus (*e.g.*, herpes simplex virus), poxvirus, baculovirus, papillomavirus, and papovavirus (*e.g.*, SV40).

Illustrative examples of expression vectors include, but are not limited to pCIneo
 30 vectors (Promega) for expression in mammalian cells; pLenti4/V5-DEST™, pLenti6/V5-

DEST™, and pLenti6.2/V5-GW/lacZ (Invitrogen) for lentivirus-mediated gene transfer and expression in mammalian cells. In particular embodiments, coding sequences of polypeptides disclosed herein can be ligated into such expression vectors for the expression of the polypeptides in mammalian cells.

5 In particular embodiments, the vector is an episomal vector or a vector that is maintained extrachromosomally. As used herein, the term “episomal” refers to a vector that is able to replicate without integration into host’s chromosomal DNA and without gradual loss from a dividing host cell also meaning that said vector replicates extrachromosomally or episomally. The vector is engineered to harbor the sequence coding for the origin of DNA
10 replication or “ori” from an alpha, beta, or gamma herpesvirus, an adenovirus, SV40, a bovine papilloma virus, or a yeast. Typically, the host cell comprises the viral replication transactivator protein that activates the replication. Alpha herpesviruses have a relatively short reproductive cycle, variable host range, efficiently destroy infected cells and establish latent infections primarily in sensory ganglia. Illustrative examples of alpha herpes viruses include
15 HSV 1, HSV 2, and VZV. Beta herpesviruses have long reproductive cycles and a restricted host range. Infected cells often enlarge. Latency can be maintained in the white cells of the blood, kidneys, secretory glands and other tissues. Illustrative examples of beta herpes viruses include CMV, HHV-6 and HHV-7. Gamma-herpesviruses are specific for either T or B lymphocytes, and latency is often demonstrated in lymphoid tissue. Illustrative examples of
20 gamma herpes viruses include EBV and HHV-8.

“Expression control sequences,” “control elements,” or “regulatory sequences” present in an expression vector are those non-translated regions of the vector—origin of replication, selection cassettes, promoters, enhancers, translation initiation signals (Shine Dalgarno sequence or Kozak sequence) introns, a polyadenylation sequence, 5' and 3' untranslated
25 regions—which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including ubiquitous promoters and inducible promoters may be used.

In particular embodiments, a polynucleotide is a vector, including but not limited to
30 expression vectors and viral vectors, and includes exogenous, endogenous, or heterologous

control sequences such as promoters and/or enhancers. An “endogenous control sequence” is one which is naturally linked with a given gene in the genome. An “exogenous control sequence” is one which is placed in juxtaposition to a gene by means of genetic manipulation (*i.e.*, molecular biological techniques) such that transcription of that gene is directed by the
5 linked enhancer/promoter. A “heterologous control sequence” is an exogenous sequence that is from a different species than the cell being genetically manipulated.

The term “promoter” as used herein refers to a recognition site of a polynucleotide (DNA or RNA) to which an RNA polymerase binds. An RNA polymerase initiates and transcribes polynucleotides operably linked to the promoter. In particular embodiments,
10 promoters operative in mammalian cells comprise an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated and/or another sequence found 70 to 80 bases upstream from the start of transcription, a CNCAAT region where N may be any nucleotide.

The term “enhancer” refers to a segment of DNA which contains sequences capable of
15 providing enhanced transcription and in some instances can function independent of their orientation relative to another control sequence. An enhancer can function cooperatively or additively with promoters and/or other enhancer elements. The term “promoter/enhancer” refers to a segment of DNA which contains sequences capable of providing both promoter and enhancer functions.

20 The term “operably linked”, refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. In one embodiment, the term refers to a functional linkage between a nucleic acid expression control sequence (such as a promoter, and/or enhancer) and a second polynucleotide sequence, *e.g.*, a polynucleotide-of-interest, wherein the expression control sequence directs transcription of the nucleic acid
25 corresponding to the second sequence.

As used herein, the term “constitutive expression control sequence” refers to a promoter, enhancer, or promoter/enhancer that continually or continuously allows for transcription of an operably linked sequence. A constitutive expression control sequence may be a “ubiquitous” promoter, enhancer, or promoter/enhancer that allows expression in a wide
30 variety of cell and tissue types or a “cell specific,” “cell type specific,” “cell lineage specific,”

or “tissue specific” promoter, enhancer, or promoter/enhancer that allows expression in a restricted variety of cell and tissue types, respectively.

Illustrative ubiquitous expression control sequences suitable for use in particular embodiments include, but are not limited to, a cytomegalovirus (CMV) immediate early promoter, a viral simian virus 40 (SV40) (*e.g.*, early or late), a Moloney murine leukemia virus (MoMLV) LTR promoter, a Rous sarcoma virus (RSV) LTR, a herpes simplex virus (HSV) (thymidine kinase) promoter, H5, P7.5, and P11 promoters from vaccinia virus, an elongation factor 1-alpha (EF1a) promoter, early growth response 1 (EGR1), ferritin H (FerH), ferritin L (FerL), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), eukaryotic translation initiation factor 4A1 (EIF4A1), heat shock 70kDa protein 5 (HSPA5), heat shock protein 90kDa beta, member 1 (HSP90B1), heat shock protein 70kDa (HSP70), β -kinesin (β -KIN), the human ROSA 26 locus (Irions *et al.*, *Nature Biotechnology* 25, 1477 - 1482 (2007)), a Ubiquitin C promoter (UBC), a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β -actin (CAG) promoter, a β -actin promoter and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter (Challita *et al.*, *J Virol.* 69(2):748-55 (1995)).

In a particular embodiment, it may be desirable to use a cell, cell type, cell lineage or tissue specific expression control sequence to achieve cell type specific, lineage specific, or tissue specific expression of a desired polynucleotide sequence (*e.g.*, to express a particular nucleic acid encoding a polypeptide in only a subset of cell types, cell lineages, or tissues or during specific stages of development).

As used herein, “conditional expression” may refer to any type of conditional expression including, but not limited to, inducible expression; repressible expression; expression in cells or tissues having a particular physiological, biological, or disease state, *etc.* This definition is not intended to exclude cell type or tissue specific expression. Certain embodiments provide conditional expression of a polynucleotide-of-interest, *e.g.*, expression is controlled by subjecting a cell, tissue, organism, *etc.*, to a treatment or condition that causes the polynucleotide to be expressed or that causes an increase or decrease in expression of the polynucleotide encoded by the polynucleotide-of-interest.

Illustrative examples of inducible promoters/systems include, but are not limited to, steroid-inducible promoters such as promoters for genes encoding glucocorticoid or estrogen receptors (inducible by treatment with the corresponding hormone), metallothioneine promoter (inducible by treatment with various heavy metals), MX-1 promoter (inducible by interferon),
 5 the “GeneSwitch” mifepristone-regulatable system (Sirin *et al.*, 2003, *Gene*, 323:67), the cumate inducible gene switch (WO 2002/088346), tetracycline-dependent regulatory systems, *etc.*

Conditional expression can also be achieved by using a site specific DNA recombinase. According to certain embodiments, polynucleotides comprises at least one (typically two)
 10 site(s) for recombination mediated by a site specific recombinase. As used herein, the terms “recombinase” or “site specific recombinase” include excisive or integrative proteins, enzymes, co-factors or associated proteins that are involved in recombination reactions involving one or more recombination sites (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more.), which may be wild-type proteins (*see* Landy, *Current Opinion in Biotechnology* 3:699-707
 15 (1993)), or mutants, derivatives (*e.g.*, fusion proteins containing the recombination protein sequences or fragments thereof), fragments, and variants thereof. Illustrative examples of recombinases suitable for use in particular embodiments include, but are not limited to: Cre, Int, IHF, Xis, Flp, Fis, Hin, Gin, Φ C31, Cin, Tn3 resolvase, TndX, XerC, XerD, TnpX, Hjc, Gin, SpCCE1, and ParA.

20 The polynucleotides may comprise one or more recombination sites for any of a wide variety of site specific recombinases. It is to be understood that the target site for a site specific recombinase is in addition to any site(s) required for integration of a vector, *e.g.*, a retroviral vector or lentiviral vector. As used herein, the terms “recombination sequence,” “recombination site,” or “site specific recombination site” refer to a particular nucleic acid
 25 sequence to which a recombinase recognizes and binds.

For example, one recombination site for Cre recombinase is loxP which is a 34 base pair sequence comprising two 13 base pair inverted repeats (serving as the recombinase binding sites) flanking an 8 base pair core sequence (*see* FIG. 1 of Sauer, B., *Current Opinion in Biotechnology* 5:521-527 (1994)). Other exemplary loxP sites include, but are not limited
 30 to: lox511 (Hoess *et al.*, 1996; Bethke and Sauer, 1997), lox5171 (Lee and Saito, 1998),

lox2272 (Lee and Saito, 1998), m2 (Langer *et al.*, 2002), lox71 (Albert *et al.*, 1995), and lox66 (Albert *et al.*, 1995).

Suitable recognition sites for the FLP recombinase include, but are not limited to: FRT (McLeod, *et al.*, 1996), F₁, F₂, F₃ (Schlake and Bode, 1994), F₄, F₅ (Schlake and Bode, 1994),
 5 FRT(LE) (Senecoff *et al.*, 1988), FRT(RE) (Senecoff *et al.*, 1988).

Other examples of recognition sequences are the attB, attP, attL, and attR sequences, which are recognized by the recombinase enzyme λ Integrase, *e.g.*, phi-c31. The ϕ C31 SSR mediates recombination only between the heterotypic sites attB (34 bp in length) and attP (39 bp in length) (Groth *et al.*, 2000). attB and attP, named for the attachment sites for the phage
 10 integrase on the bacterial and phage genomes, respectively, both contain imperfect inverted repeats that are likely bound by ϕ C31 homodimers (Groth *et al.*, 2000). The product sites, attL and attR, are effectively inert to further ϕ C31-mediated recombination (Belteki *et al.*, 2003), making the reaction irreversible. For catalyzing insertions, it has been found that attB-bearing DNA inserts into a genomic attP site more readily than an attP site into a genomic attB site
 15 (Thyagarajan *et al.*, 2001; Belteki *et al.*, 2003). Thus, typical strategies position by homologous recombination an attP-bearing “docking site” into a defined locus, which is then partnered with an attB-bearing incoming sequence for insertion.

In one embodiment, a polynucleotide contemplated herein comprises a repair template polynucleotide flanked by a pair of recombinase recognition sites. In particular embodiments,
 20 the repair template polynucleotide is flanked by LoxP sites, FRT sites, or att sites.

In particular embodiments, polynucleotides contemplated herein, include one or more polynucleotides-of-interest that encode one or more polypeptides. In particular embodiments, to achieve efficient translation of each of the plurality of polypeptides, the polynucleotide sequences can be separated by one or more IRES sequences or polynucleotide sequences
 25 encoding self-cleaving polypeptides.

As used herein, an “internal ribosome entry site” or “IRES” refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent translation of the gene. *See, e.g.*, Jackson *et al.*, 1990. *Trends Biochem Sci* 15(12):477-83) and Jackson and Kaminski.
 30 1995. *RNA* 1(10):985-1000. Examples of IRES generally employed by those of skill in the art

include those described in U.S. Pat. No. 6,692,736. Further examples of “IRES” known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson *et al.*, 1990) and IRES obtainable from viral or cellular mRNA sources, such as for example, immunoglobulin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez *et al.*, 1998. *Mol. Cell. Biol.* 18(11):6178-6190), the fibroblast growth factor 2 (FGF-2), and insulin-like growth factor (IGFII), the translational initiation factor eIF4G and yeast transcription factors TFIID and HAP4, the encephelomyocarditis virus (EMCV) which is commercially available from Novagen (Duke *et al.*, 1992. *J. Virol* 66(3):1602-9) and the VEGF IRES (Huez *et al.*, 1998. *Mol Cell Biol* 18(11):6178-90). IRES have also been reported in viral genomes of Picornaviridae, Dicistroviridae and Flaviviridae species and in HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV).

In one embodiment, the IRES used in polynucleotides contemplated herein is an EMCV IRES.

In particular embodiments, the polynucleotides comprise a consensus Kozak sequence. As used herein, the term “Kozak sequence” refers to a short nucleotide sequence that greatly facilitates the initial binding of mRNA to the small subunit of the ribosome and increases translation. The consensus Kozak sequence is (GCC)RCCATGG (SEQ ID NO:50), where R is a purine (A or G) (Kozak, 1986. *Cell.* 44(2):283-92, and Kozak, 1987. *Nucleic Acids Res.* 15(20):8125-48).

Elements directing the efficient termination and polyadenylation of the heterologous nucleic acid transcripts increases heterologous gene expression. Transcription termination signals are generally found downstream of the polyadenylation signal. In particular embodiments, vectors comprise a polyadenylation sequence 3' of a polynucleotide encoding a polypeptide to be expressed. The term “polyA site” or “polyA sequence” as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript by RNA polymerase II. Polyadenylation sequences can promote mRNA stability by addition of a polyA tail to the 3' end of the coding sequence and thus, contribute to increased translational efficiency. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a polyA tail are unstable and are rapidly degraded. Illustrative examples of polyA signals that can be used in a vector, includes an ideal polyA sequence (*e.g.*,

AATAAA, ATTAAA, AGTAAA), a bovine growth hormone polyA sequence (BGHpA), a rabbit β -globin polyA sequence (r β gpA), or another suitable heterologous or endogenous polyA sequence known in the art.

In some embodiments, a polynucleotide or cell harboring the polynucleotide utilizes a suicide gene, including an inducible suicide gene to reduce the risk of direct toxicity and/or uncontrolled proliferation. In specific embodiments, the suicide gene is not immunogenic to the host harboring the polynucleotide or cell. A certain example of a suicide gene that may be used is caspase-9 or caspase-8 or cytosine deaminase. Caspase-9 can be activated using a specific chemical inducer of dimerization (CID).

In certain embodiments, polynucleotides comprise gene segments that cause the genetically modified cells contemplated herein to be susceptible to negative selection *in vivo*. "Negative selection" refers to an infused cell that can be eliminated as a result of a change in the *in vivo* condition of the individual. The negative selectable phenotype may result from the insertion of a gene that confers sensitivity to an administered agent, for example, a compound. Negative selection genes are known in the art, and include, but are not limited to: the Herpes simplex virus type I thymidine kinase (HSV-I TK) gene which confers ganciclovir sensitivity; the cellular hypoxanthine phosphoribosyltransferase (HPRT) gene, the cellular adenine phosphoribosyltransferase (APRT) gene, and bacterial cytosine deaminase.

In some embodiments, genetically modified cells comprise a polynucleotide further comprising a positive marker that enables the selection of cells of the negative selectable phenotype *in vitro*. The positive selectable marker may be a gene, which upon being introduced into the host cell, expresses a dominant phenotype permitting positive selection of cells carrying the gene. Genes of this type are known in the art, and include, but are not limited to hygromycin-B phosphotransferase gene (hph) which confers resistance to hygromycin B, the amino glycoside phosphotransferase gene (neo or aph) from Tn5 which codes for resistance to the antibiotic G418, the dihydrofolate reductase (DHFR) gene, the adenosine deaminase gene (ADA), and the multi-drug resistance (MDR) gene.

In one embodiment, the positive selectable marker and the negative selectable element are linked such that loss of the negative selectable element necessarily also is accompanied by loss of the positive selectable marker. In a particular embodiment, the positive and negative

selectable markers are fused so that loss of one obligatorily leads to loss of the other. An example of a fused polynucleotide that yields as an expression product a polypeptide that confers both the desired positive and negative selection features described above is a hygromycin phosphotransferase thymidine kinase fusion gene (HyTK). Expression of this
 5 gene yields a polypeptide that confers hygromycin B resistance for positive selection *in vitro*, and ganciclovir sensitivity for negative selection *in vivo*. See also the publications of PCT US91/08442 and PCT/US94/05601, by S. D. Lupton, describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable markers with negative selectable markers.

10 Preferred positive selectable markers are derived from genes selected from the group consisting of hph, nco, and gpt, and preferred negative selectable markers are derived from genes selected from the group consisting of cytosine deaminase, HSV-I TK, VZV TK, HPRT, APRT and gpt. Exemplary bifunctional selectable fusion genes contemplated in particular embodiments include, but are not limited to genes wherein the positive selectable marker is
 15 derived from hph or neo, and the negative selectable marker is derived from cytosine deaminase or a TK gene or selectable marker.

In particular embodiments, polynucleotides encoding one or more meganucleases, megaTALs, TALENs, ZFNs, Cas nucleases, end-processing nucleases, CARs, therapeutic polypeptides, and fusion polypeptides may be introduced into immune effector cells, *e.g.*, T
 20 cells, by both non-viral and viral methods. In particular embodiments, delivery of one or more polynucleotides encoding nucleases and/or donor repair templates may be provided by the same method or by different methods, and/or by the same vector or by different vectors.

The term “vector” is used herein to refer to a nucleic acid molecule capable transferring
 25 or transporting another nucleic acid molecule. The transferred nucleic acid is generally linked to, *e.g.*, inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication in a cell, or may include sequences sufficient to allow integration into host cell DNA. In particular embodiments, non-viral vectors are used to deliver one or more polynucleotides contemplated herein to a T cell.

Illustrative examples of non-viral vectors include, but are not limited to plasmids (*e.g.*, DNA plasmids or RNA plasmids), transposons, cosmids, bacterial artificial chromosomes, and viral vectors.

5 Illustrative methods of non-viral delivery of polynucleotides contemplated in particular embodiments include, but are not limited to: electroporation, sonoporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, nanoparticles, polycation or lipid:nucleic acid conjugates, naked DNA, artificial virions, DEAE-dextran-mediated transfer, gene gun, and heat-shock.

10 Illustrative examples of polynucleotide delivery systems suitable for use in particular embodiments contemplated in particular embodiments include, but are not limited to those provided by Amaxa Biosystems, Maxcyte, Inc., BTX Molecular Delivery Systems, and Copernicus Therapeutics Inc. Lipofection reagents are sold commercially (*e.g.*, Transfectam™ and Lipofectin™). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides have been described in the literature. See 15 *e.g.*, Liu *et al.* (2003) *Gene Therapy*. 10:180–187; and Balazs *et al.* (2011) *Journal of Drug Delivery*. 2011:1-12. Antibody-targeted, bacterially derived, non-living nanocell-based delivery is also contemplated in particular embodiments.

Viral vectors comprising polynucleotides contemplated in particular embodiments can be delivered *in vivo* by administration to an individual patient, typically by systemic 20 administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.*, mobilized peripheral blood, lymphocytes, bone marrow aspirates, tissue biopsy, *etc.*) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient.

25 In one embodiment, viral vectors comprising engineered nucleases and/or donor repair templates are administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. 30 Suitable methods of administering such nucleic acids are available and well known to those

of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Illustrative examples of viral vector systems suitable for use in particular
5 embodiments contemplated in particular embodiments include, but are not limited to adeno-associated virus (AAV), retrovirus, herpes simplex virus, adenovirus, vaccinia virus vectors for gene transfer.

In various embodiments, one or more polynucleotides encoding an engineered
nuclease and/or donor repair template are introduced into an immune effector cell, *e.g.*, T
10 cell, by transducing the cell with a recombinant adeno-associated virus (rAAV), comprising the one or more polynucleotides.

AAV is a small (~26 nm) replication-defective, primarily episomal. non-enveloped virus. AAV can infect both dividing and non-dividing cells and may incorporate its genome into that of the host cell. Recombinant AAV (rAAV) are typically composed of, at a
15 minimum, a transgene and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). The ITR sequences are about 145 bp in length. In particular embodiments, the rAAV comprises ITRs and capsid sequences isolated from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10.

In some embodiments, a chimeric rAAV is used the ITR sequences are isolated from
20 one AAV serotype and the capsid sequences are isolated from a different AAV serotype. For example, a rAAV with ITR sequences derived from AAV2 and capsid sequences derived from AAV6 is referred to as AAV2/AAV6. In particular embodiments, the rAAV vector may comprise ITRs from AAV2, and capsid proteins from any one of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10. In a preferred
25 embodiment, the rAAV comprises ITR sequences derived from AAV2 and capsid sequences derived from AAV6.

In some embodiments, engineering and selection methods can be applied to AAV capsids to make them more likely to transduce cells of interest.

Construction of rAAV vectors, production, and purification thereof have been disclosed, *e.g.*, in U.S. Patent Nos. 9,169,494; 9,169,492; 9,012,224; 8,889,641; 8,809,058; and 8,784,799, each of which is incorporated by reference herein, in its entirety.

In various embodiments, one or more polynucleotides encoding a CAR, an engineered nuclease, or donor repair template are introduced into an immune effector cell, *e.g.*, T cell, by transducing the cell with a retrovirus, *e.g.*, lentivirus, comprising the one or more polynucleotides.

As used herein, the term “retrovirus” refers to an RNA virus that reverse transcribes its genomic RNA into a linear double-stranded DNA copy and subsequently covalently integrates its genomic DNA into a host genome. Illustrative retroviruses suitable for use in particular embodiments, include, but are not limited to: Moloney murine leukemia virus (M-MuLV), Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), spumavirus, Friend murine leukemia virus, Murine Stem Cell Virus (MSCV) and Rous Sarcoma Virus (RSV)) and lentivirus.

As used herein, the term “lentivirus” refers to a group (or genus) of complex retroviruses. Illustrative lentiviruses include, but are not limited to: HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2); visna-maedi virus (VMV) virus; the caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV). In one embodiment, HIV based vector backbones (*i.e.*, HIV cis-acting sequence elements) are preferred.

In various embodiments, a lentiviral vector contemplated herein comprises one or more LTRs, and one or more, or all, of the following accessory elements: a cPPT/FLAP, a Psi (Ψ) packaging signal, an export element, poly (A) sequences, and may optionally comprise a WPRE or HPRE, an insulator element, a selectable marker, and a cell suicide gene, as discussed elsewhere herein.

In particular embodiments, lentiviral vectors contemplated herein may be integrative or non-integrating or integration defective lentivirus. As used herein, the term “integration defective lentivirus” refers to a lentivirus having an integrase that lacks the capacity to integrate

the viral genome into the genome of the host cells. Integration-incompetent viral vectors have been described in patent application WO 2006/010834, which is herein incorporated by reference in its entirety.

Illustrative mutations in the HIV-1 pol gene suitable to reduce integrase activity
 5 include, but are not limited to: H12N, H12C, H16C, H16V, S81 R, D41A, K42A, H51A, Q53C, D55V, D64E, D64V, E69A, K71A, E85A, E87A, D116N, D116I, D116A, N120G, N120I, N120E, E152G, E152A, D35E, K156E, K156A, E157A, K159E, K159A, K160A, R166A, D167A, E170A, H171A, K173A, K186Q, K186T, K188T, E198A, R199c, R199T, R199A, D202A, K211A, Q214L, Q216L, Q221 L, W235F, W235E, K236S, K236A, K246A,
 10 G247W, D253A, R262A, R263A and K264H.

The term “long terminal repeat (LTR)” refers to domains of base pairs located at the ends of retroviral DNAs which, in their natural sequence context, are direct repeats and contain U3, R and U5 regions.

As used herein, the term “FLAP element” or “cPPT/FLAP” refers to a nucleic acid
 15 whose sequence includes the central polypurine tract and central termination sequences (cPPT and CTS) of a retrovirus, *e.g.*, HIV-1 or HIV-2. Suitable FLAP elements are described in U.S. Pat. No. 6,682,907 and in Zennou, *et al.*, 2000, *Cell*, 101:173.

As used herein, the term “packaging signal” or “packaging sequence” refers to psi [Ψ] sequences located within the retroviral genome which are required for insertion of the viral
 20 RNA into the viral capsid or particle, *see e.g.*, Clever *et al.*, 1995. *J. of Virology*, Vol. 69, No. 4; pp. 2101–2109.

The term “export element” refers to a cis-acting post-transcriptional regulatory element which regulates the transport of an RNA transcript from the nucleus to the cytoplasm of a cell. Examples of RNA export elements include, but are not limited to, the human
 25 immunodeficiency virus (HIV) rev response element (RRE) (*see e.g.*, Cullen *et al.*, 1991. *J. Virol.* 65: 1053; and Cullen *et al.*, 1991. *Cell* 58: 423), and the hepatitis B virus post-transcriptional regulatory element (HPRE).

In particular embodiments, expression of heterologous sequences in viral vectors is increased by incorporating posttranscriptional regulatory elements, efficient polyadenylation
 30 sites, and optionally, transcription termination signals into the vectors. A variety of

posttranscriptional regulatory elements can increase expression of a heterologous nucleic acid at the protein, *e.g.*, woodchuck hepatitis virus posttranscriptional regulatory element (WPRE; Zufferey *et al.*, 1999, *J. Virol.*, 73:2886); the posttranscriptional regulatory element present in hepatitis B virus (HPRE) (Huang *et al.*, *Mol. Cell. Biol.*, 5:3864); and the like (Liu *et al.*, 1995, 5 *Genes Dev.*, 9:1766).

Lentiviral vectors preferably contain several safety enhancements as a result of modifying the LTRs. “Self-inactivating” (SIN) vectors refers to replication-defective vectors, *e.g.*, in which the right (3') LTR enhancer-promoter region, known as the U3 region, has been modified (*e.g.*, by deletion or substitution) to prevent viral transcription beyond the first round 10 of viral replication. An additional safety enhancement is provided by replacing the U3 region of the 5' LTR with a heterologous promoter to drive transcription of the viral genome during production of viral particles. Examples of heterologous promoters which can be used include, for example, viral simian virus 40 (SV40) (*e.g.*, early or late), cytomegalovirus (CMV) (*e.g.*, immediate early), Moloney murine leukemia virus (MoMLV), Rous sarcoma virus (RSV), and 15 herpes simplex virus (HSV) (thymidine kinase) promoters.

The terms “pseudotype” or “pseudotyping” as used herein, refer to a virus whose viral envelope proteins have been substituted with those of another virus possessing preferable characteristics. For example, HIV can be pseudotyped with vesicular stomatitis virus G-protein (VSV-G) envelope proteins, which allows HIV to infect a wider range of 20 cells because HIV envelope proteins (encoded by the *env* gene) normally target the virus to CD4⁺ presenting cells.

In certain embodiments, lentiviral vectors are produced according to known methods. *See e.g.*, Kutner *et al.*, *BMC Biotechnol.* 2009;9:10. doi: 10.1186/1472-6750-9-10; Kutner *et al.* *Nat. Protoc.* 2009;4(4):495–505. doi: 10.1038/nprot.2009.22.

25 According to certain specific embodiments contemplated herein, most or all of the viral vector backbone sequences are derived from a lentivirus, *e.g.*, HIV-1. However, it is to be understood that many different sources of retroviral and/or lentiviral sequences can be used, or combined and numerous substitutions and alterations in certain of the lentiviral sequences may be accommodated without impairing the ability of a transfer vector to 30 perform the functions described herein. Moreover, a variety of lentiviral vectors are known

in the art, *see* Naldini *et al.*, (1996a, 1996b, and 1998); Zufferey *et al.*, (1997); Dull *et al.*, 1998, U.S. Pat. Nos. 6,013,516; and 5,994,136, many of which may be adapted to produce a viral vector or transfer plasmid contemplated herein.

In various embodiments, one or more polynucleotides encoding a CAR, an
5 engineered nuclease, or donor repair template are introduced into an immune effector cell, by transducing the cell with an adenovirus comprising the one or more polynucleotides.

Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and high levels of expression have been obtained. This vector can be produced in large quantities in a relatively
10 simple system. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and/or E3 genes; subsequently the replication defective vector is propagated in human 293 cells that supply deleted gene function in trans. Ad vectors can transduce multiple types of tissues *in vivo*, including non-dividing, differentiated cells such as those found in liver, kidney and muscle. Conventional Ad vectors have a large carrying capacity.

15 Generation and propagation of the current adenovirus vectors, which are replication deficient, may utilize a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the adenovirus genome (Jones & Shenk, 1978), the current adenovirus vectors, with the help of 293 cells,
20 carry foreign DNA in either the E1, the D3 or both regions (Graham & Prevec, 1991). Adenovirus vectors have been used in eukaryotic gene expression (Levrero *et al.*, 1991; Gomez-Foix *et al.*, 1992) and vaccine development (Grunhaus & Horwitz, 1992; Graham & Prevec, 1992). Studies in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld *et al.*, 1991; Rosenfeld *et al.*, 1992), muscle injection (Ragot *et al.*, 1993), peripheral intravenous injections (Herz & Gerard, 1993) and stereotactic
25 inoculation into the brain (Le Gal La Salle *et al.*, 1993). An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Serman *et al.*, *Hum. Gene Ther.* 7:1083-9 (1998)).

In various embodiments, one or more polynucleotides encoding a CAR, an engineered
30 nuclease, or donor repair template are introduced into an immune effector cell by transducing

the cell with a herpes simplex virus, *e.g.*, HSV-1, HSV-2, comprising the one or more polynucleotides.

The mature HSV virion consists of an enveloped icosahedral capsid with a viral genome consisting of a linear double-stranded DNA molecule that is 152 kb. In one embodiment, the HSV based viral vector is deficient in one or more essential or non-essential HSV genes. In one embodiment, the HSV based viral vector is replication deficient. Most replication deficient HSV vectors contain a deletion to remove one or more intermediate-early, early, or late HSV genes to prevent replication. For example, the HSV vector may be deficient in an immediate early gene selected from the group consisting of: ICP4, ICP22, ICP27, ICP47, and a combination thereof. Advantages of the HSV vector are its ability to enter a latent stage that can result in long-term DNA expression and its large viral DNA genome that can accommodate exogenous DNA inserts of up to 25 kb. HSV-based vectors are described in, for example, U.S. Pat. Nos. 5,837,532, 5,846,782, and 5,804,413, and International Patent Applications WO 91/02788, WO 96/04394, WO 98/15637, and WO 99/06583, each of which are incorporated by reference herein in its entirety.

H. COMPOSITIONS AND FORMULATIONS

The compositions contemplated in particular embodiments may comprise one or more polypeptides, polynucleotides, vectors comprising same, and immune effector cell compositions, as contemplated herein. Compositions include, but are not limited to pharmaceutical compositions. A “pharmaceutical composition” refers to a composition formulated in pharmaceutically-acceptable or physiologically-acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. It will also be understood that, if desired, the compositions may be administered in combination with other agents as well, such as, *e.g.*, cytokines, growth factors, hormones, small molecules, chemotherapeutics, pro-drugs, drugs, antibodies, or other various pharmaceutically-active agents. There is virtually no limit to other components that may also be included in the compositions, provided that the additional agents do not adversely affect the ability of the composition to deliver the intended therapy.

The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

As used herein “pharmaceutically acceptable carrier, diluent or excipient” includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, surfactant, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals. Exemplary pharmaceutically acceptable carriers include, but are not limited to, to sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; tragacanth; malt; gelatin; talc; cocoa butter, waxes, animal and vegetable fats, paraffins, silicones, bentonites, silicic acid, zinc oxide; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and any other compatible substances employed in pharmaceutical formulations.

In particular embodiments, compositions comprise an amount genome edited CAR T cells manufactured by the methods contemplated herein. In preferred embodiments, the pharmaceutical compositions comprise CAR T cells comprising one or more modified and/or non-functional TCR α alleles and that express one or more CARs.

It can generally be stated that a pharmaceutical composition comprising the CAR T cells manufactured by the methods contemplated in particular embodiments may be administered at a dosage of about 10^2 to about 10^{10} cells/kg body weight, about 10^5 to about 10^9 cells/kg body weight, about 10^5 to about 10^8 cells/kg body weight, about 10^5 to about 10^7 cells/kg body weight, about 10^7 to about 10^9 cells/kg body weight, or about 10^7 to about 10^8

cells/kg body weight, including all integer values within those ranges. The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 mL or less, even 250 mL or 100 mL or less. Hence the density of the desired cells is typically greater than about 10^6 cells/mL and generally is greater than about 10^7 cells/mL, generally about 10^8 cells/mL or greater. The clinically relevant number of immune cells can be apportioned into multiple infusions that cumulatively equal or exceed about 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , or 10^{12} cells.

In some embodiments, particularly since all the infused cells will be redirected to a particular target antigen, lower numbers of cells, in the range of 10^6 /kilogram (10^6 - 10^{11} per patient) may be administered. T cells modified to express a CAR may be administered multiple times at dosages within these ranges. The cells may be allogeneic, syngeneic, xenogeneic, or autologous to the patient undergoing therapy. If desired, the treatment may also include administration of mitogens (*e.g.*, PHA) or lymphokines, cytokines, and/or chemokines (*e.g.*, IFN- γ , IL-2, IL-7, IL-15, IL-12, TNF-alpha, IL-18, and TNF-beta, GM-CSF, IL-4, IL-13, Flt3-L, RANTES, MIP1 α , *etc.*) as described herein to enhance engraftment and function of infused T cells.

Generally, compositions comprising the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, compositions comprising the modified T cells manufactured by the methods contemplated herein are used in the treatment of cancer. The genome edited CAR T cells contemplated in particular embodiments may be administered either alone, or as a pharmaceutical composition in combination with carriers, diluents, excipients, and/or with other components such as IL-2, IL-7, and/or IL-15 or other cytokines or cell populations. In particular embodiments, pharmaceutical compositions contemplated herein comprise an amount of CAR T cells comprising one or more modified TCR α alleles, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients.

Pharmaceutical compositions comprising genome edited CAR T cells contemplated in particular embodiments may further comprise buffers such as neutral buffered saline,

phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions contemplated in particular embodiments are preferably
5 formulated for parenteral administration, *e.g.*, intravascular (intravenous or intraarterial), intraperitoneal or intramuscular administration.

The liquid pharmaceutical compositions, whether they be solutions, suspensions or other like form, may include one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium
10 chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium
15 chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. An injectable pharmaceutical composition is preferably sterile.

In one embodiment, the genome edited CAR T cell compositions are formulated in a pharmaceutically acceptable cell culture medium. Such compositions are suitable for
20 administration to human subjects. In particular embodiments, the pharmaceutically acceptable cell culture medium is a serum free medium.

Serum-free medium has several advantages over serum containing medium, including a simplified and better defined composition, a reduced degree of contaminants, elimination of a potential source of infectious agents, and lower cost. In various embodiments, the serum-free
25 medium is animal-free, and may optionally be protein-free. Optionally, the medium may contain biopharmaceutically acceptable recombinant proteins. "Animal-free" medium refers to medium wherein the components are derived from non-animal sources. Recombinant proteins replace native animal proteins in animal-free medium and the nutrients are obtained from synthetic, plant or microbial sources. "Protein-free" medium, in contrast, is defined as
30 substantially free of protein.

Illustrative examples of serum-free media used in particular compositions includes, but is not limited to QBSF-60 (Quality Biological, Inc.), StemPro-34 (Life Technologies), and X-VIVO 10.

In one preferred embodiment, compositions comprising genome edited CAR T cells
5 contemplated herein are formulated in a solution comprising PlasmaLyte A.

In another preferred embodiment, compositions comprising genome edited CAR T cells contemplated herein are formulated in a solution comprising a cryopreservation medium. For example, cryopreservation media with cryopreservation agents may be used to maintain a high cell viability outcome post-thaw. Illustrative examples of cryopreservation media used in
10 particular compositions includes, but is not limited to, CryoStor CS10, CryoStor CS5, and CryoStor CS2.

In a more preferred embodiment, compositions comprising genome edited CAR T cells contemplated herein are formulated in a solution comprising 50:50 PlasmaLyte A to CryoStor CS10.

15 In a particular embodiment, compositions contemplated herein comprise an effective amount of an expanded genome edited CAR T cell composition, alone or in combination with one or more therapeutic agents. Thus, the CAR T cell compositions may be administered alone or in combination with other known cancer treatments, such as radiation therapy, chemotherapy, transplantation, immunotherapy, hormone therapy, photodynamic therapy, *etc.*
20 The compositions may also be administered in combination with antibiotics. Such therapeutic agents may be accepted in the art as a standard treatment for a particular disease state as described herein, such as a particular cancer. Exemplary therapeutic agents contemplated in particular embodiments include cytokines, growth factors, steroids, NSAIDs, DMARDs, anti-inflammatories, chemotherapeutics, radiotherapeutics, therapeutic antibodies, or other active
25 and ancillary agents.

In certain embodiments, compositions comprising CAR T cells contemplated herein may be administered in conjunction with any number of chemotherapeutic agents. Illustrative examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclophosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and
30 piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa;

ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, anthramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiolesterol, mepitiolesterol, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2''-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®) and doxetaxel (TAXOTERE®); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000;

difluoromethylomithine (DMFO); retinoic acid derivatives such as Targretin™ (bexarotene), Panretin™ (alitretinoin); ONTAK™ (denileukin diftitox); esperamicins; capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors
 5 such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

10 A variety of other therapeutic agents may be used in conjunction with the compositions contemplated herein. In one embodiment, the composition comprising CAR T cells is administered with an anti-inflammatory agent. Anti-inflammatory agents or drugs include, but are not limited to, steroids and glucocorticoids (including betamethasone, budesonide, dexamethasone, hydrocortisone acetate, hydrocortisone, hydrocortisone, methylprednisolone,
 15 prednisolone, prednisone, triamcinolone), nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, ibuprofen, naproxen, methotrexate, sulfasalazine, leflunomide, anti-TNF medications, cyclophosphamide and mycophenolate.

Other exemplary NSAIDs are chosen from the group consisting of ibuprofen, naproxen, naproxen sodium, Cox-2 inhibitors such as VIOXX® (rofecoxib) and
 20 CELEBREX® (celecoxib), and sialylates. Exemplary analgesics are chosen from the group consisting of acetaminophen, oxycodone, tramadol or propoxyphene hydrochloride. Exemplary glucocorticoids are chosen from the group consisting of cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisolone, or prednisone. Exemplary biological response modifiers include molecules directed against cell surface markers (*e.g.*, CD4, CD5,
 25 *etc.*), cytokine inhibitors, such as the TNF antagonists (*e.g.*, etanercept (ENBREL®), adalimumab (HUMIRA®) and infliximab (REMICADE®), chemokine inhibitors and adhesion molecule inhibitors. The biological response modifiers include monoclonal antibodies as well as recombinant forms of molecules. Exemplary DMARDs include
 30 sulfasalazine, hydroxychloroquine, Gold (oral (auranofin) and intramuscular) and minocycline.

Illustrative examples of therapeutic antibodies suitable for combination with the genome edited T cells contemplated in particular embodiments, include but are not limited to, abagovomab, adecatumumab, afutuzumab, alemtuzumab, altumomab, amatuximab, anatumomab, arcitumomab, bavituximab, bectumomab, bevacizumab, bivatuzumab, blinatumomab, brentuximab, cantuzumab, catumaxomab, cetuximab, citatuzumab, cixutumumab, clivatuzumab, conatumumab, daratumumab, drozitumab, duligotumab, dusigitumab, detumomab, dacetuzumab, dalotuzumab, ecomeximab, elotuzumab, ensituximab, ertumaxomab, etaracizumab, farietuzumab, ficlatuzumab, figitumumab, flinvotumab, futuximab, ganitumab, gemtuzumab, girentuximab, glembatumumab, ibritumomab, igovomab, imgatuzumab, indatuximab, inotuzumab, intetumumab, ipilimumab, iratumumab, labetuzumab, lexatumumab, lintuzumab, lorvotuzumab, lucatumumab, mapatumumab, matuzumab, milatuzumab, minretumomab, mitumomab, moxetumomab, narnatumab, naptumomab, necitumumab, nimotuzumab, nofetumomab, ocaratuzumab, ofatumumab, olaratumab, onartuzumab, oportuzumab, oregovomab, panitumumab, parsatuzumab, patritumab, pentumomab, pertuzumab, pintumomab, pritumumab, racotumomab, radretumab, rilotumumab, rituximab, robatumumab, satumomab, sibrotuzumab, siltuximab, simtuzumab, solitomab, tacatuzumab, taplitumomab, tenatumomab, teprotumumab, tigatuzumab, tositumomab, trastuzumab, tucotuzumab, ublituximab, veltuzumab, vorsetuzumab, votumumab, zalutumumab, CC49 and 3E8.

In certain embodiments, the compositions contemplated herein are administered in conjunction with a cytokine. By “cytokine” as used herein is meant a generic term for proteins released by one cell population that act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, chemokines, and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin;

thrombopoietin (TPO); nerve growth factors such as NGF-beta; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, beta, and -gamma; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-15, a tumor necrosis factor such as TNF-alpha or TNF-beta; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture, and biologically active equivalents of the native sequence cytokines.

I. TARGET CELLS

In particular embodiments, it is contemplated that genome edited CAR T cells redirected to a target cell, *e.g.*, a tumor or cancer cell, have a binding domain that binds to target antigens on the cells.

In one embodiment, the target cell expresses an antigen, *e.g.*, a target antigen that is not substantially found on the surface of other normal (desired) cells.

In one embodiment, the target cell is a bone cell, osteocyte, osteoblast, adipose cell, chondrocyte, chondroblast, muscle cell, skeletal muscle cell, myoblast, myocyte, smooth muscle cell, bladder cell, bone marrow cell, central nervous system (CNS) cell, peripheral nervous system (PNS) cell, glial cell, astrocyte cell, neuron, pigment cell, epithelial cell, skin cell, endothelial cell, vascular endothelial cell, breast cell, colon cell, esophagus cell, gastrointestinal cell, stomach cell, colon cell, head cell, neck cell, gum cell, tongue cell, kidney cell, liver cell, lung cell, nasopharynx cell, ovary cell, follicular cell, cervical cell, vaginal cell, uterine cell, pancreatic cell, pancreatic parenchymal cell, pancreatic duct cell, pancreatic islet cell, prostate cell, penile cell, gonadal cell, testis cell, hematopoietic cell, lymphoid cell, or myeloid cell.

In one embodiment, the target cell is solid cancer cell.

Illustrative examples of cells that can be targeted by the compositions and methods contemplated in particular embodiments include, but are not limited to those of the following

solid cancers: adrenal cancer, adrenocortical carcinoma, anal cancer, appendix cancer, astrocytoma, atypical teratoid/rhabdoid tumor, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain/CNS cancer, breast cancer, bronchial tumors, cardiac tumors, cervical cancer, cholangiocarcinoma, chondrosarcoma, chordoma, colon cancer, colorectal cancer, craniopharyngioma, ductal carcinoma in situ (DCIS) endometrial cancer, ependymoma, 5 esophageal cancer, esthesioneuroblastoma, Ewing's sarcoma, extracranial germ cell tumor, extragonadal germ cell tumor, eye cancer, fallopian tube cancer, fibrous histiosarcoma, fibrosarcoma, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (GIST), germ cell tumors, glioma, glioblastoma, head and neck cancer, hemangioblastoma, hepatocellular cancer, hypopharyngeal cancer, intraocular 10 melanoma, kaposi sarcoma, kidney cancer, laryngeal cancer, leiomyosarcoma, lip cancer, liposarcoma, liver cancer, lung cancer, non-small cell lung cancer, lung carcinoid tumor, malignant mesothelioma, medullary carcinoma, medulloblastoma, meningioma, melanoma, Merkel cell carcinoma, midline tract carcinoma, mouth cancer, myxosarcoma, myelodysplastic syndrome, myeloproliferative neoplasms, nasal cavity and paranasal sinus cancer, 15 nasopharyngeal cancer, neuroblastoma, oligodendroglioma, oral cancer, oral cavity cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic islet cell tumors, papillary carcinoma, paraganglioma, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pinealoma, pituitary tumor, pleuropulmonary blastoma, primary peritoneal cancer, prostate cancer, rectal cancer, retinoblastoma, renal cell carcinoma, renal 20 pelvis and ureter cancer, rhabdomyosarcoma, salivary gland cancer, sebaceous gland carcinoma, skin cancer, soft tissue sarcoma, squamous cell carcinoma, small cell lung cancer, small intestine cancer, stomach cancer, sweat gland carcinoma, synovioma, testicular cancer, throat cancer, thymus cancer, thyroid cancer, urethral cancer, uterine cancer, uterine sarcoma, vaginal cancer, vascular cancer, vulvar cancer, and Wilms Tumor. 25

In one embodiment, the target cell is liquid cancer or hematological cancer cell.

Illustrative examples of hematological cancers include, but are not limited to: leukemias, lymphomas, and multiple myeloma.

Illustrative examples of cells that can be targeted by the compositions and methods 30 contemplated in particular embodiments include, but are not limited to those of the following

leukemias: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML) and polycythemia vera.

5 Illustrative examples of cells that can be targeted by the compositions and methods contemplated in particular embodiments include, but are not limited to those of the following lymphomas: Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma and Non-Hodgkin lymphoma, including but not limited to B-cell non-Hodgkin lymphomas: Burkitt lymphoma, small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma,
10 follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, marginal zone lymphoma, and mantle cell lymphoma; and T-cell non-Hodgkin lymphomas: mycosis fungoides, anaplastic large cell lymphoma, Sézary syndrome, and precursor T-lymphoblastic lymphoma. .

Illustrative examples of cells that can be targeted by the compositions and methods
15 contemplated in particular embodiments include, but are not limited to those of the following multiple myelomas: overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

In another particular embodiment, the target cell is a cancer cell, such as a cell in a
20 patient with cancer.

In one embodiment, the target cell is a cell, *e.g.*, a cancer cell infected by a virus, including but not limited to CMV, HPV, and EBV.

In one embodiment, the target antigen is an epitope of alpha folate receptor, 5T4, $\alpha_v\beta_6$ integrin, BCMA, B7-H3, B7-H6, CAIX, CD19, CD20, CD22, CD30, CD33, CD44,
25 CD44v6, CD44v7/8, CD70, CD79a, CD79b, CD123, CD138, CD171, CEA, CSPG4, EGFR, EGFR family including ErbB2 (HER2), EGFRvIII, EGP2, EGP40, EPCAM, EphA2, EpCAM, FAP, fetal AchR, FR α , GD2, GD3, Glypican-3 (GPC3), HLA-A1+MAGE1, HLA-A2+MAGE1, HLA-A3+MAGE1, HLA-A1+NY-ESO-1, HLA-A2+NY-ESO-1, HLA-A3+NY-ESO-1, IL-11R α , IL-13R α 2, Lambda, Lewis-Y, Kappa,

Mesothelin, Muc1, Muc16, NCAM, NKG2D Ligands, NY-ESO-1, PRAME, PSCA, PSMA, ROR1, SSX, Survivin, TAG72, TEMs, VEGFR2, and WT-1.

J. THERAPEUTIC METHODS

The genome edited CAR T cells manufactured by the compositions and methods contemplated herein provide improved adoptive cell therapy for use in the treatment of various conditions including, without limitation, cancer, infectious disease, autoimmune disease, inflammatory disease, and immunodeficiency. In particular embodiments, the specificity of a primary T cell is redirected to tumor or cancer cells by genetically modifying the primary T cell with a CAR contemplated herein. In one embodiment, the genome edited CAR T cell is infused to a recipient in need thereof. The infused cell is able to kill tumor cells in the recipient.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of solid tumors or cancers.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of solid tumors or cancers including, but not limited to: adrenal cancer, adrenocortical carcinoma, anal cancer, appendix cancer, astrocytoma, atypical teratoid/rhabdoid tumor, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain/CNS cancer, breast cancer, bronchial tumors, cardiac tumors, cervical cancer, cholangiocarcinoma, chondrosarcoma, chordoma, colon cancer, colorectal cancer, craniopharyngioma, ductal carcinoma in situ (DCIS) endometrial cancer, ependymoma, esophageal cancer, esthesioneuroblastoma, Ewing's sarcoma, extracranial germ cell tumor, extragonadal germ cell tumor, eye cancer, fallopian tube cancer, fibrous histiosarcoma, fibrosarcoma, gallbladder cancer, gastric cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor (GIST), germ cell tumors, glioma, glioblastoma, head and neck cancer, hemangioblastoma, hepatocellular cancer, hypopharyngeal cancer, intraocular melanoma, kaposi sarcoma, kidney cancer, laryngeal cancer, leiomyosarcoma, lip cancer, liposarcoma, liver cancer, lung cancer, non-small cell lung cancer, lung carcinoid tumor, malignant mesothelioma, medullary carcinoma, medulloblastoma, meningioma, melanoma, Merkel cell carcinoma, midline tract carcinoma, mouth cancer, myxosarcoma, myelodysplastic

syndrome, myeloproliferative neoplasms, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, oligodendroglioma, oral cancer, oral cavity cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic islet cell tumors, papillary carcinoma, paraganglioma, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pinealoma, pituitary tumor, pleuropulmonary blastoma, primary peritoneal cancer, prostate cancer, rectal cancer, retinoblastoma, renal cell carcinoma, renal pelvis and ureter cancer, rhabdomyosarcoma, salivary gland cancer, sebaceous gland carcinoma, skin cancer, soft tissue sarcoma, squamous cell carcinoma, small cell lung cancer, small intestine cancer, stomach cancer, sweat gland carcinoma, synovioma, testicular cancer, throat cancer, thymus cancer, thyroid cancer, urethral cancer, uterine cancer, uterine sarcoma, vaginal cancer, vascular cancer, vulvar cancer, and Wilms Tumor.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of solid tumors or cancers including, without limitation, liver cancer, pancreatic cancer, lung cancer, breast cancer, bladder cancer, brain cancer, bone cancer, thyroid cancer, kidney cancer, or skin cancer.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of various cancers including but not limited to pancreatic, bladder, and lung.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of liquid cancers or hematological cancers.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of B-cell malignancies, including but not limited to: leukemias, lymphomas, and multiple myeloma.

In particular embodiments, genome edited CAR T cells contemplated herein are used in the treatment of liquid cancers including, but not limited to the following leukemias, lymphomas, and multiple myelomas: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML) and polycythemia vera, Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, Burkitt lymphoma, small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma, follicular

lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell lymphoma, marginal zone lymphoma, mycosis fungoides, anaplastic large cell lymphoma, Sézary syndrome, precursor T-lymphoblastic lymphoma, multiple myeloma, overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD
 5 myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

In particular embodiments, methods comprising administering a therapeutically effective amount of genome edited T cells contemplated herein or a composition comprising the same, to a patient in need thereof, alone or in combination with one or more therapeutic
 10 agents, are provided. In certain embodiments, the cells are used in the treatment of patients at risk for developing a cancer. Thus, particular embodiments comprise the treatment or prevention or amelioration of at least one symptom of a cancer comprising administering to a subject in need thereof, a therapeutically effective amount of the genome edited T cells contemplated herein.

15 In one embodiment, a method of treating a cancer in a subject in need thereof comprises administering an effective amount, *e.g.*, therapeutically effective amount of a composition comprising genome edited CAR T cells contemplated herein. The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be
 20 determined by clinical trials.

In one embodiment, the amount of T cells in the composition administered to a subject is at least 0.1×10^5 cells, at least 0.5×10^5 cells, at least 1×10^5 cells, at least 5×10^5 cells, at least 1×10^6 cells, at least 0.5×10^7 cells, at least 1×10^7 cells, at least 0.5×10^8 cells, at least 1×10^8 cells, at least 0.5×10^9 cells, at least 1×10^9 cells, at least 2×10^9 cells, at least 3×10^9
 25 cells, at least 4×10^9 cells, at least 5×10^9 cells, or at least 1×10^{10} cells.

In particular embodiments, about 1×10^7 T cells to about 1×10^9 T cells, about 2×10^7 T cells to about 0.9×10^9 T cells, about 3×10^7 T cells to about 0.8×10^9 T cells, about 4×10^7 T cells to about 0.7×10^9 T cells, about 5×10^7 T cells to about 0.6×10^9 T cells, or about 5×10^7 T cells to about 0.5×10^9 T cells are administered to a subject.

In one embodiment, the amount of T cells in the composition administered to a subject is at least 0.1×10^4 cells/kg of bodyweight, at least 0.5×10^4 cells/kg of bodyweight, at least 1×10^4 cells/kg of bodyweight, at least 5×10^4 cells/kg of bodyweight, at least 1×10^5 cells/kg of bodyweight, at least 0.5×10^6 cells/kg of bodyweight, at least 1×10^6 cells/kg of bodyweight, at least 0.5×10^7 cells/kg of bodyweight, at least 1×10^7 cells/kg of bodyweight, at least 0.5×10^8 cells/kg of bodyweight, at least 1×10^8 cells/kg of bodyweight, at least 2×10^8 cells/kg of bodyweight, at least 3×10^8 cells/kg of bodyweight, at least 4×10^8 cells/kg of bodyweight, at least 5×10^8 cells/kg of bodyweight, or at least 1×10^9 cells/kg of bodyweight.

In particular embodiments, about 1×10^6 T cells/kg of bodyweight to about 1×10^8 T cells/kg of bodyweight, about 2×10^6 T cells/kg of bodyweight to about 0.9×10^8 T cells/kg of bodyweight, about 3×10^6 T cells/kg of bodyweight to about 0.8×10^8 T cells/kg of bodyweight, about 4×10^6 T cells/kg of bodyweight to about 0.7×10^8 T cells/kg of bodyweight, about 5×10^6 T cells/kg of bodyweight to about 0.6×10^8 T cells/kg of bodyweight, or about 5×10^6 T cells/kg of bodyweight to about 0.5×10^8 T cells/kg of bodyweight are administered to a subject.

One of ordinary skill in the art would recognize that multiple administrations of the compositions contemplated in particular embodiments may be required to effect the desired therapy. For example a composition may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more times over a span of 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 1 year, 2 years, 5 years, 10 years, or more.

In certain embodiments, it may be desirable to administer activated T cells to a subject and then subsequently redraw blood (or have an apheresis performed), activate T cells therefrom, and reinfuse the patient with these activated and expanded T cells. This process can be carried out multiple times every few weeks. In certain embodiments, T cells can be activated from blood draws of from 10cc to 400cc. In certain embodiments, T cells are activated from blood draws of 20cc, 30cc, 40cc, 50cc, 60cc, 70cc, 80cc, 90cc, 100cc, 150cc, 200cc, 250cc, 300cc, 350cc, or 400cc or more. Not to be bound by theory, using this multiple blood draw/multiple reinfusion protocol may serve to select out certain populations of T cells.

The administration of the compositions contemplated in particular embodiments may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion,

transfusion, implantation or transplantation. In a preferred embodiment, compositions are administered parenterally. The phrases “parenteral administration” and “administered parenterally” as used herein refers to modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravascular, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intratumoral, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion. In one embodiment, the compositions contemplated herein are administered to a subject by direct injection into a tumor, lymph node, or site of infection.

10 In one embodiment, a subject in need thereof is administered an effective amount of a composition to increase a cellular immune response to a cancer in the subject. The immune response may include cellular immune responses mediated by cytotoxic T cells capable of killing infected cells, regulatory T cells, and helper T cell responses. Humoral immune responses, mediated primarily by helper T cells capable of activating B cells thus leading to antibody production, may also be induced. A variety of techniques may be used for analyzing the type of immune responses induced by the compositions, which are well described in the art; *e.g.*, Current Protocols in Immunology, Edited by: John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober (2001) John Wiley & Sons, NY, N.Y.

20 In one embodiment, a method of treating a subject diagnosed with a cancer, comprises removing immune effector cells from the subject, editing the genome of said immune effector cells and producing a population of genome edited immune effector cells, introducing a nucleic acid encoding a CAR into the cells, and administering the population of genome edited immune effector cells to the same subject. In a preferred embodiment, the immune effector cells comprise T cells.

25 The methods for administering the cell compositions contemplated in particular embodiments include any method which is effective to result in reintroduction of *ex vivo* genome edited immune effector cells or on reintroduction of the genome edited progenitors of immune effector cells that on introduction into a subject differentiate into mature immune effector cells. One method comprises genome editing peripheral blood T cells

and introducing a nucleic acid encoding a CAR into the cells *ex vivo* and returning the transduced cells into the subject.

5 All publications, patent applications, and issued patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or issued patent were specifically and individually indicated to be incorporated by reference.

10 Although the foregoing embodiments have been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings contemplated herein that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

EXAMPLE 1ANTI-BCMA CAR T CELLS WITH A DISRUPTED TCR α ALLELE*Culture Methods*

5 Cryopreserved peripheral blood mononuclear cells (PBMCs) were isolated from two distinct healthy donors using a Ficoll gradient, thawed, and placed into culture with anti-CD3 and anti-CD28 T-cell stimulating antibodies. Twenty four hours post-antibody stimulation, cells were transduced at an MOI of 10 with a lentivirus encoding a second generation chimeric antigen receptor (CAR) comprising an anti-BCMA scFv, a 4-1BB co-stimulatory domain, and
10 a CD3 ζ signaling domain. Seventy two hours post-antibody stimulation, 100×10^6 transduced cells (large scale) or 5×10^6 transduced cells (small scale) were electroporated using a Maxcyte electroporation device with 50 μ g/mL of *in vitro* transcribed mRNA encoding a megaTAL that targets the TCR α locus. Cells were placed back into culture immediately following electroporation and incubated at 30°C for 24 hours, returned to a 37°C incubator, and cultured
15 in fresh media containing IL-2 for a total of ten days. On day 10, cells were cryopreserved for future analysis.

Vector Copy Number Analysis

The number of integrated lentiviral particles was determined by q-PCR and presented as vector copy number (VCN). VCN was determined by isolating genomic DNA from cells
20 using commercially available gDNA isolation kits, performing a quantitative PCR assay with taqMan probes for Psi/Gag regions of the integrated provirus, and comparing the Psi/Gag signal to endogenous RNaseP copy numbers using a standard curve generated with a plasmid-based positive control. VCNs were similar for each donor for anti-BCMA CAR T cells cultured using the standard culture process (no EP), cells that were electroporated but received
25 no mRNA (EP only), and cells electroporated with mRNA encoding a megaTAL that targets the TCR α gene (TCR α KO). Figure 1.

Cell Growth

5 Viable cell counts were performed on both donors during the 10 day culture process using a Nexcelom counting device and trypan blue exclusion to determine the impact of the electroporation protocol on the cell expansion. Cells that were electroporated alone (EP only) or electroporated with TCR α megaTAL mRNA (TCR α KO) and incubated at 30°C overnight exhibited a delayed expansion relative to cells cultured using the standard process, but resumed similar growth kinetics between days 5 and 10 post PBMC culture initiation. Figure 2.

Anti-BCMA CAR Expression and TCR α Targeting Efficiency (CD3 negative cells)

10 Anti-BCMA CAR expression and efficiency of TCR α disruption via flow cytometric analysis were determined at day 10. CAR expression was determined by staining cells with BCMA antigen conjugated to PE fluorophore. Anti-BCMA CAR expression was similar among the electroporated and non-electroporated groups. Figure 3, right-most quadrants. megaTAL mediated TCR α disruption was determined by CD3 surface staining. Detectable CD3 expression requires the TCR α chain trafficking to the cell surface and has previously been
15 shown to correlate well with genetic analysis of TCR α disruption. The data show that CD3 is removed from the surface of ~50% of T cells electroporated with megaTAL mRNA at large scale, and ~60% of cells at small scale. Figure 3, lower-most quadrants. TCR α disruption was similar between CAR positive and CAR negative cells. Figure 3, compare lower left quadrant (CAR negative) to lower right quadrant (CAR positive). Figure 4 shows a graphical
20 representation of the FACS data shown in Figure 3.

Antigen Dependent Cytokine Release

The biological activity of anti-BCMA CAR T cells with a disrupted TCR α allele (TCR α KO) to BCMA-positive and BCMA-negative cell lines was assessed using an interferon-gamma (IFN γ) release assay. 5 x 10⁴ T cells were expanded using the standard
25 process (no EP), EP only, and TCR α KO as described above were co-cultured with BCMA positive and BCMA negative cell lines at a 1:1 ratio for 24 hours at 37°C. Culture media supernatants were collected and standard luminex assays were performed to assess relative cytokine production from the different cell types. TCR α KO cells secreted higher levels of the key immunostimulatory cytokine IFN γ in an antigen dependent manner. Figure 5A.

The biological activity of anti-BCMA CAR T cells with a disrupted TCR α allele (TCR α KO +/- Trex2) to BCMA-positive and BCMA-negative cell lines was assessed using a cytokine release assay. 5×10^4 T cells were expanded using the standard process (no EP), TCR α KO, or TCR α KO generated with Trex 2. Anti-BCMA CAR T cells were co-cultured T cells, or with BCMA positive and BCMA negative cell lines at a 1:1 ratio for 24 hours at 37°C. Culture media supernatants were collected and standard luminex assays were performed to assess relative IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α production from the different cell types. All anti-BCMA CAR T cells produced cytokines in an antigen dependent manner. Figure 5B.

10 *In vivo Efficacy of TCR α KO Cells*

Anti-BCMA CAR T cells manufactured using the standard process (no EP), EP only, and TCR α KO treatments described above were tested for *in vivo* efficacy using a Daudi tumor xenograft model. 2×10^6 Daudi cells, which constitutively express luciferase, were injected into NSG mice and allowed to engraft and grow until luciferase signals reached $\sim 1 \times 10^7$ p/sec/cm²/sr (about 11 days post tumor cell injection) as assessed by a Xenogen IVIS imager. At this time, mice were injected with 10×10^6 , 5×10^6 , or 2.5×10^6 anti-BCMA CAR T cells (n=5 per group). Tumor growth was followed by serial imaging of mice twice a week via Xenogen imaging of luciferase. At 27 days, TCR α KO cells, across all doses, outperformed anti-BCMA CAR T cells cultured using the standard process and those which were electroporated without mRNA. Figure 6A.

TCR α KO cells continue to outperform other anti-BCMA CAR T cells at day 43. Figure 6B.

EXAMPLE 2

TCR α DISRUPTION ENHANCES CYTOKINE RELEASE FROM ANTI-CD19 CAR T CELLS

Antigen dependent cytokine production in T cells transduced with a lentivirus encoding an anti-CD19 CAR was compared to antigen dependent cytokine production in genome edited T cells containing an anti-CD19 CAR integrated into the TCR α locus.

Lentiviral anti-CD19 CAR

Human PBMCs (1×10^6 cells/mL) were activated with soluble anti-CD3 and anti-CD28 antibodies (50 ng/mL) on day 0. After 24hr incubation, 1×10^6 cells were transduced with an anti-CD19 CAR lentivirus (LV-T cells; pBB146, SEQ ID NO: 8). The lentiviral vector
 5 contained a CAR expression cassette comprising an MND promoter operably linked to a CAR comprising a CD8 α -derived signal peptide, an anti-CD19 scFv, a CD8 α derived hinge region and transmembrane domain, an intracellular 4-1BB co-stimulatory domain, and a CD3 ζ signaling domain. Lentivirus was prepared using established protocols. *See e.g., Kutner et al., BMC Biotechnol.* 2009;9:10. doi: 10.1186/1472-6750-9-10; Kutner *et al. Nat. Protoc.*
 10 2009;4(4):495–505. doi: 10.1038/nprot.2009.22.

Anti-CD19 CAR Integrated into the TCR α Locus

Adeno-associated virus (AAV) plasmids containing an anti-CD19 CAR was designed, constructed, and verified (pBW1021, SEQ ID NO: 9). The donor repair template contained a CAR expression cassette comprising an MND promoter operably linked to a CAR comprising
 15 a CD8 α -derived signal peptide, an anti-CD19 scFv, a CD8 α derived hinge region and transmembrane domain, an intracellular 4-1BB co-stimulatory domain, and a CD3 ζ signaling domain. The 5' and 3' homology regions of the donor repair template were about 650 bp each.

Recombinant AAV (rAAV) was prepared by transiently co-transfecting HEK 293T cells with one or more plasmids providing the replication, capsid, and adenoviral helper
 20 elements necessary. rAAV was purified from the co-transfected HEK 293T cell culture using ultracentrifugation in an iodixanol-based gradient.

Human PBMCs (1×10^6 cells/mL) were activated with soluble anti-CD3 and anti-CD28 antibodies (50 ng/mL) on day 0. On day 3 post-activation, cells were electroporated with buffer only (UTD control) or mRNA encoding a TCR α -targeting megaTAL polypeptide
 25 (SEQ ID NO: 11). Electroporated cells were incubated for one hour at 30°C and transduced with rAAV encoding an anti-CD19 CAR donor repair template to drive homologous recombination (HR-T cells) at the TCR α locus. The genome edited cells were incubated at 30°C for 24 hours and then were recovered and cultured at 37°C for 5 days with IL-2 containing medium.

Anti-CD19 CAR Expression and TCR α Targeting Efficiency (CD3 negative cells)

On day 10 post-activation, anti-CD19 CAR T cells were analyzed by flow cytometry to detect CD3 and anti-CD19 CAR surface expression. Lentiviral transduction generated 72% CD3⁺ anti-CD19-CAR⁺ T cells, whereas 28-40% of HR-T cells were CD3⁻ anti-CD19 CAR⁺.

5 Figure 7.

Antigen Dependent Cytotoxicity

Cytotoxic potential of LV-T and HR-T cells was analyzed by co-incubating T cells with 50:50 mixtures of the CD19⁺ Nalm-6 (GFP) and K562 (BFP) target cells for 24 hours at an effector to target (E:T) ratio of 1:1. The ratio of Nalm-6 vs. K562 following T cell co-
 10 culture is a direct readout of T cell cytotoxicity. Similar levels of CD19 specific cytotoxicity was observed in both LV-T and HR-T cell co-cultures. Figure 8A.

Antigen Dependent Cytokine Release

Antigen dependent cytokine release was analyzed by co-culturing LV-T cells or HR-T cells with CD19⁺ Nalm-6 cells for 24 hours at 1: 1 E:T ratio. Culture supernatants were
 15 collected and IFN γ release was analyzed using the Cytometric Bead Array (CBA) (BD biosciences). HR-T cells released increased amounts of IFN γ compared to LV-T cells. Figure 8B.

EXAMPLE 3

TCR α DISRUPTION ENHANCES CYTOKINE RELEASE
 20 FROM ANTI-CD19 CAR T CELLS

Antigen dependent cytokine production in T cells transduced with a lentivirus encoding an anti-CD19 CAR was compared to antigen dependent cytokine production in genome edited T cells containing an anti-CD19 CAR integrated into the TCR α locus.

Lentiviral anti-CD19 CAR

25 Human PBMCs (1×10^6 cells/mL) were activated with soluble anti-CD3 and anti-CD28 antibodies (50 ng/mL) on day 0. After 24hr incubation, 1×10^6 cells were transduced with an anti-CD19 CAR lentivirus (LV-T cells; pBB146, SEQ ID NO: 8). The lentiviral

vector contained a CAR expression cassette comprising an MND promoter operably linked to a CAR comprising a CD8 α -derived signal peptide, an anti-CD19 scFv, a CD8 α derived hinge region and transmembrane domain, an intracellular 4-1BB co-stimulatory domain, and a CD3 ζ signaling domain. Lentivirus was prepared using established protocols. *See e.g., Kutner et al., BMC Biotechnol.* 2009;9:10. doi: 10.1186/1472-6750-9-10; Kutner et al. *Nat. Protoc.* 2009;4(4):495–505. doi: 10.1038/nprot.2009.22.

Anti-CD19 CAR Integrated into the TCR α Locus

Adeno-associated virus (AAV) plasmids containing an anti-CD19 CAR was designed, constructed, and verified (pBW400, SEQ ID NO: 13). The donor repair template contained a CAR expression cassette comprising an MND promoter operably linked to a CAR comprising a CD8 α -derived signal peptide, an anti-CD19 scFv, a CD8 α derived hinge region and transmembrane domain, an intracellular 4-1BB co-stimulatory domain, and a CD3 ζ signaling domain, and a woodchuck post-transcriptional regulatory region (WPRE). The 5' and 3' homology regions of the donor repair template were about 650 bp each.

Recombinant AAV (rAAV) was prepared by transiently co-transfecting HEK 293T cells with one or more plasmids providing the replication, capsid, and adenoviral helper elements necessary. rAAV was purified from the co-transfected HEK 293T cell culture using ultracentrifugation in an iodixanol-based gradient.

Human PBMCs (1×10^6 cells/mL) were activated with soluble anti-CD3 and anti-CD28 antibodies (50 ng/mL) on day 0. On day 3 post-activation, cells were electroporated with buffer only (UTD control) or mRNA encoding a TCR α -targeting megaTAL polypeptide (SEQ ID NO: 11). Electroporated cells were incubated for one hour at 30°C and transduced with rAAV encoding an anti-CD19 CAR donor repair template to drive homologous recombination (HR-T cells) at the TCR α locus. The genome edited cells were incubated at 30°C for 24 hours and then were recovered and cultured at 37°C for 5 days with IL-2 containing medium.

Anti-CD19 CAR Expression and TCR α Targeting Efficiency (CD3 negative cells)

On day 10 post-activation, anti-CD19 CAR T cells were analyzed by flow cytometry to detect CD3 and anti-CD19 CAR surface expression. Lentiviral transduction generated 85%

CD3⁺ anti-CD19-CAR⁺ T cells, whereas 49% of HR-T cells were CD3⁺ anti-CD19 CAR⁺.
Figure 9.

Antigen Dependent Cytotoxicity

Cytotoxic potential of LV-T and HR-T cells was analyzed by co-incubating T cells
5 with 50:50 mixtures of the CD19⁺ Nalm-6 (GFP) and K562 (BFP) target cells for 24 hours at
an effector to target (E:T) ratio of 1:1. The ratio of Nalm-6 vs. K562 following T cell co-
culture is a direct readout of T cell cytotoxicity. Similar levels of CD19 specific cytotoxicity
was observed in both LV-T and HR-T cell co-cultures. Figure 10A.

Antigen Dependent Cytokine Release

10 Antigen dependent cytokine release was analyzed by co-culturing LV-T cells or HR-T
cells with CD19⁺ Nalm-6 cells for 24 hours at 1: 1 E:T ratio. Culture supernatants were
collected and IFN γ , IL-2, IL-4, and TNF α release were analyzed using the Cytometric Bead
Array (CBA) (BD biosciences). HR-T cells released increased amounts of cytokines compared
to LV-T cells. Figure 10B.

15

EXAMPLE 4

HOMOLOGOUS RECOMBINATION OF AN ANTI-CD19 CAR TRANSGENE INTO THE TCRA LOCUS IS ASSOCIATED WITH REDUCED EXPRESSION OF T CELL EXHAUSTION MARKERS

T cell exhaustion in T cells transduced with a lentivirus encoding an anti-CD19 CAR
20 was compared to T cell exhaustion in genome edited T cells containing an anti-CD19 CAR
integrated into the TCR α locus.

An adeno-associated virus (AAV) plasmid containing a promoter, a transgene encoding
anti-CD19 CAR, and a polyadenylation signal, were designed, constructed, and verified.
Recombinant AAV (rAAV) was prepared by transiently co-transfecting HEK 293T cells with
25 one or more plasmids providing the replication, capsid, and adenoviral helper elements
necessary. rAAV was purified from the co-transfected HEK 293T cell culture using
ultracentrifugation in an iodixanol-based gradient.

The lentiviral vector contained a CAR expression cassette comprising an MND promoter operably linked to a CAR comprising a CD8 α -derived signal peptide, an anti-CD19 scFv, a CD8 α derived hinge region and transmembrane domain, an intracellular 4-1BB co-stimulatory domain, and a CD3 ζ signaling domain. Lentivirus was prepared using established
5 protocols. *See e.g., Kutner et al., BMC Biotechnol.* 2009;9:10. doi: 10.1186/1472-6750-9-10; Kutner et al. *Nat. Protoc.* 2009;4(4):495–505. doi: 10.1038/nprot.2009.22.

Primary human T cells were activated with CD3 and CD28, as described in Example 1. Activated primary human T cells were electroporated with *in vitro* transcribed megaTAL mRNA and transduced with rAAV targeting vectors encoding the anti-CD19 CAR transgene
10 (HR-CAR T cells); or activated primary human T cells were transduced with a lentivirus encoding an anti-CD19 CAR (LV-CAR T cells).

LV-T and HR-T cells were co-cultured with CD19 expressing Nalm-6 cells in 1:1 Effector (E) cell to Target (T) cell ratio. T cell exhaustion marker expression (PD-L1, PD-1, and Tim-3) was measured at 24 hours and 72 hours of co-culture. At 24 hours, HR-CAR T
15 cells showed reduced upregulation of PD-1 and PD-L1 compared to LV-CAR T cells. Figure 11A. At 72 hours, HR-CAR T cells showed reduced upregulation of PD-1 and Tim-3 compared to LV-CAR T cells. Figure 11B.

20 In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A chimeric antigen receptor (CAR) T cell, comprising, one or more modified T cell receptor alpha (TCR α) alleles, wherein the CAR T cell secretes an increased amount of proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.
2. The CAR T cell of claim 1, wherein the CAR comprises an extracellular domain that binds a target antigen selected from the group consisting of: BCMA, CD19, CSPG4, PSCA, ROR1, and TAG72.
3. The CAR T cell of claim 1 or claim 2, wherein the CAR comprises a transmembrane domain isolated from a polypeptide selected from the group consisting of: alpha or beta chain of the T-cell receptor, CD3 δ , CD3 ϵ , CD3 γ , CD3 ζ , CD4, CD5, CD8 α , CD9, CD 16, CD22, CD27, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD 134, CD137, CD152, CD154, and PD-1.
4. The CAR T cell of any one of claims 1-3, wherein the CAR comprises one or more intracellular costimulatory signaling domains isolated from a polypeptide selected from the group consisting of: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, CARD11, CD2, CD7, CD27, CD28, CD30, CD40, CD54 (ICAM), CD83, CD134 (OX40), CD137 (4-1BB), CD278 (ICOS), DAP10, LAT, NKD2C, SLP76, TRIM, and ZAP70.
5. The CAR T cell of any one of claim 1-4, wherein the CAR comprises a signaling domain isolated from a polypeptide selected from the group consisting of: FcR γ , FcR β , CD3 γ , CD3 δ , CD3 ϵ , CD3 ζ , CD22, CD79a, CD79b, and CD66d.

6. The CAR T cell of any one of claim 1-5, wherein the CAR comprises a hinge region polypeptide selected from the group consisting of: a hinge region of CD8 α , a hinge region of PD1, and a hinge region of CD152.

7. The CAR T cell of any one of claim 1-6, wherein the CAR comprises one or more linker polypeptides.

8. The CAR T cell of any one of claim 1-7, wherein the CAR comprises a spacer region polypeptide that comprises CH2 and CH3 regions of IgG1, IgG4, or IgD.

9. The CAR T cell of any one of claim 1-8, wherein the CAR comprises a signal peptide selected from the group consisting of: an IgG1 heavy chain signal polypeptide, a CD8 α signal polypeptide, and a human GM-CSF receptor alpha signal polypeptide.

10. The CAR T cell of any one of claims 1-9, wherein the one or more modified TCR α alleles are non-functional or have substantially reduced function.

11. The CAR T cell of any one of claims 1-10, wherein the CAR T cell comprises one or more proviral integrants comprising a nucleic acid encoding the CAR.

12. The CAR T cell of any one of claims 1-10, wherein the CAR T cell comprises a polynucleotide comprising a promoter operably linked to a nucleic acid encoding the CAR, wherein the polynucleotide has been inserted into the one or more TCR α alleles by homology directed repair.

13. The CAR T cell of any one of claims 1-12, wherein the one or more proinflammatory cytokines are selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α .

14. A CAR T cell, comprising:
a) one or more modified TCR α alleles; and

b) a nucleic acid encoding a CAR that binds a target antigen,
 wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when contacted with a target cell expressing the target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

15. A CAR T cell, comprising:
 a) one or more modified TCR α alleles; and
 b) one or more proviral integrants comprising a nucleic acid encoding a CAR that binds a target antigen,
 wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

16. A CAR T cell, comprising, one or more modified TCR α alleles, wherein a donor repair template comprising a nucleic acid encoding a CAR that binds a target antigen is inserted into the one or more TCR α alleles by homology directed repair, and wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks one or more modified TCR α alleles.

17. The CAR T cell of any one of claims 14-16, wherein the nucleic acid further comprises an RNA polymerase II promoter operably linked to the polynucleotide encoding the CAR.

18. The CAR T cell of claim 17, wherein the RNA polymerase II promoter is selected from the group consisting of: a short EF1 α promoter, a long EF1 α promoter, a human ROSA 26 locus, a Ubiquitin C (UBC) promoter, a phosphoglycerate kinase-1 (PGK) promoter, a cytomegalovirus enhancer/chicken β -actin (CAG) promoter, a β -actin promoter and a myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted (MND) promoter.

19. The CAR T cell of any one of claims 14-18, wherein the CAR comprises an extracellular domain that binds an antigen selected from the group consisting of: BCMA, CD19, CSPG4, PSCA, ROR1, and TAG72.

20. The CAR T cell of any one of claims 14-19, wherein the CAR further comprises a transmembrane domain and one or more intracellular signaling domains.

21. The CAR T cell of any one of claims 14-20, wherein the CAR comprises a transmembrane domain, a costimulatory domain, and a primary signaling domain.

22. The CAR T cell of any one of claims 14-21, wherein the CAR further comprises a signal peptide, one or more linker peptides, and one or more hinge peptides, and optionally one or more spacer peptides.

23. The CAR T cell of any one of claims 14-21, wherein the CAR comprises an anti-BCMA scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

24. The CAR T cell of any one of claims 14-21, wherein the CAR comprises an anti-CD19 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

25. The CAR T cell of any one of claims 14-21, wherein the CAR comprises an anti-CSPG4 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

26. The CAR T cell of any one of claims 14-21, wherein the CAR comprises an anti-ROR1 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

27. The CAR T cell of any one of claims 14-21, wherein the CAR comprises an anti-PSCA scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

28. The CAR T cell of any one of claims 14-21, wherein the CAR comprises an anti-TAG72 scFv, a CD8 α transmembrane domain, a CD137 costimulatory domain, and a CD3 ζ primary signaling domain.

29. The CAR T cell of any one of claims 14-28, wherein the one or more proinflammatory cytokines are selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α .

30. A composition comprising the CAR T cell of any one of claims 1-29.

31. A composition comprising the CAR T cell of any one of claims 1-29 and a physiologically acceptable excipient.

32. A method of making a CAR T cell comprising:

a) engineering a double-strand break at a target site in a TCR α allele, wherein the break is repaired by non-homologous end joining (NHEJ), thereby generating a modified TCR α allele; and

b) transducing the T cell with a viral vector encoding a CAR;

wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks a modified TCR α allele.

33. The method of claim 32, wherein the modified TCR α allele is non-functional or has substantially reduced function.

34. The method of claim 32 or claim 33, wherein the viral vector is a retroviral vector.

35. The method of claim 34, wherein the retroviral vector is a lentiviral vector.
36. A method of making a CAR T cell comprising:
- a) engineering a double-strand break at a target site in a TCR α allele; and
 - b) transducing the T cell with a viral vector comprising a donor repair template that comprises an RNA polymerase II promoter operably linked to a nucleic acid encoding a CAR; wherein the donor repair template is incorporated into the TCR α allele by homology directed repair at the site of the double-strand break (DSB); and wherein the CAR T cell secretes an increased amount of one or more proinflammatory cytokines when bound to a target antigen compared to a CAR T cell bound to the target antigen that lacks a modified TCR α allele.
37. The method of claim 36, wherein the donor repair template comprises a 5' homology arm homologous to the TCR α sequence 5' of the DSB; and a 3' homology arm homologous to the TCR α sequence 3' of the DSB.
38. The method of claim 36 or 37, wherein the viral vector is a recombinant adeno-associated viral vector (rAAV) or a retrovirus.
39. The method of claim 38, wherein the rAAV has one or more ITRs from AAV2.
40. The method of claim 38 or claim 39, wherein the rAAV has a serotype selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and AAV10.
41. The method of claim 38, wherein the retrovirus is a lentivirus.
42. The method of claim 41, wherein the lentivirus is an integrase deficient lentivirus.
43. The method of any one of claims 32-42, wherein the double-strand break is generated with an engineered nuclease.

44. The method of claim 42, wherein the engineered nuclease is selected from the group consisting of: a meganuclease, a megaTAL, a TALEN, a ZFN, or a CRISPR/Cas nuclease.

45. The method of claim 43 or claim 44, wherein the meganuclease is engineered from an I-OnuI LHE.

46. The method of claim 43 or claim 44, wherein the engineered nuclease is a megaTAL comprising a TALE DNA binding domain and an engineered meganuclease.

47. The method of any one of claims 32-46, wherein the double-strand break is generated by an engineered endonuclease and an end-processing enzyme.

48. The method of claim 47, wherein an mRNA encoding the engineered endonuclease and an mRNA encoding the end-processing enzyme are introduced into the T cell to generate the double-strand break.

49. The method of claim 47, wherein an mRNA encoding the engineered endonuclease, a viral-self cleaving peptide, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

50. The method of claim 47, wherein an mRNA encoding the engineered endonuclease, an IRES element, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

51. The method of claim 47, wherein an mRNA encoding the engineered endonuclease fused to an end-processing enzyme or biologically active fragment thereof, are introduced into the T cell to generate the double-strand break.

52. The method of any one of claims 47 to 51 wherein the end-processing enzyme exhibits 5'-3' exonuclease, 5'-3' alkaline exonuclease, 3'-5'exonuclease, 5' flap endonuclease, helicase or template-independent DNA polymerases activity.

53. The method of any one of claims 47-52, wherein the end-processing enzyme comprises Trex2 or a biologically active fragment thereof.

54. The method of any one of claims 32-53, wherein the one or more proinflammatory cytokines are selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α .

55. A method of treating a cancer in a subject comprising, administering a CAR T cell according to any one of claims 1-29, or a composition of claim 30 or claim 31 to the subject.

56. The method of claim 55, wherein a therapeutically effective amount of CAR T cells comprising a modified TCR α allele that is administered to the subject to clear the tumor is less than the therapeutically effective amount of CAR T cells lacking a modified TCR α that is administered to the subject to clear the tumor.

57. The method of claim 55 or claim 56, wherein the cancer is a solid cancer.

58. The method of claim 55 or claim 56, wherein the cancer is a liquid cancer.

59. The method of claim 58, wherein the liquid cancer is a hematological malignancy selected from the group consisting of: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, hairy cell leukemia (HCL), chronic lymphocytic leukemia (CLL), and chronic myeloid leukemia (CML), chronic myelomonocytic leukemia (CMML) and polycythemia vera, Hodgkin lymphoma, nodular lymphocyte-predominant Hodgkin lymphoma, Burkitt lymphoma, small lymphocytic lymphoma (SLL), diffuse large B-cell lymphoma, follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, mantle cell

lymphoma, marginal zone lymphoma, mycosis fungoides, anaplastic large cell lymphoma, Sézary syndrome, precursor T-lymphoblastic lymphoma, multiple myeloma, overt multiple myeloma, smoldering multiple myeloma, plasma cell leukemia, non-secretory myeloma, IgD myeloma, osteosclerotic myeloma, solitary plasmacytoma of bone, and extramedullary plasmacytoma.

60. The method of claim 56, wherein the hematological malignancy is multiple myeloma.

61. The method of claim 56, wherein the hematological malignancy is CLL.

62. A method of treating a B cell related condition in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of CAR T cells according to any one of claims 1-29, or a therapeutically effective amount of a composition of claim 30 or claim 31 to the subject.

63. The method of claim 62, wherein the B cell related condition is multiple myeloma, non-Hodgkin's lymphoma, B cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, post-transplant lymphoproliferative disorder, an immunoregulatory disorder, rheumatoid arthritis, myasthenia gravis, idiopathic thrombocytopenia purpura, anti-phospholipid syndrome, Chagas' disease, Grave's disease, Wegener's granulomatosis, poly-arteritis nodosa, Sjogren's syndrome, pemphigus vulgaris, scleroderma, multiple sclerosis, anti-phospholipid syndrome, ANCA associated vasculitis, Goodpasture's disease, Kawasaki disease, autoimmune hemolytic anemia, and rapidly progressive glomerulonephritis, heavy-chain disease, primary or immunocyte-associated amyloidosis, or monoclonal gammopathy of undetermined significance.

64. The method of claim 63, wherein the B cell related condition is a B cell malignancy.

65. The method of claim 63, wherein the B cell related condition is a plasma cell malignancy.

66. The method of claim 63, wherein the B cell related condition is an autoimmune disease.

67. A method of increasing the efficacy of a CAR T cell comprising decreasing the availability of TCR α signaling components on the CAR T cell surface by introducing an engineered nuclease that creates a double-strand break (DSB) at a target site in a TCR α allele to modify the TCR α allele.

68. The method of claim 67, wherein the CAR T cell comprises one or more proviral integrants encoding the CAR and wherein the DSB is repaired by non-homologous end joining (NHEJ).

69. The method of claim 67, wherein the CAR T cell comprises an RNA polymerase II promoter operably linked to a nucleic acid encoding a CAR incorporated into the TCR α allele by homology directed repair at the DSB.

70. The method of any one of claims 67-69, wherein the engineered nuclease is selected from the group consisting of: a meganuclease, a megaTAL, a TALEN, a ZFN, or a CRISPR/Cas nuclease.

71. The method of claim 70, wherein the meganuclease is engineered from an I-OnuI LHE.

72. The method of claim 70, wherein the engineered nuclease is a megaTAL comprising a TALE DNA binding domain and an engineered meganuclease.

73. The method of any one of claims 67-69, wherein the double-strand break is generated by an engineered endonuclease and an end-processing enzyme.

74. The method of claim 73, wherein an mRNA encoding the engineered endonuclease and an mRNA encoding the end-processing enzyme are introduced into the T cell to generate the double-strand break.

75. The method of claim 73, wherein an mRNA encoding the engineered endonuclease, a viral-self cleaving peptide, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

76. The method of claim 73, wherein an mRNA encoding the engineered endonuclease, an IRES element, and an end-processing enzyme are introduced into the T cell to generate the double-strand break.

77. The method of claim 73, wherein an mRNA encoding the engineered endonuclease fused to an end-processing enzyme or biologically active fragment thereof, are introduced into the T cell to generate the double-strand break.

78. The method of any one of claims 74-77, wherein the end-processing enzyme exhibits 5-3' exonuclease, 5-3' alkaline exonuclease, 3-5' exonuclease, 5' flap endonuclease, helicase or template-independent DNA polymerases activity.

79. The method of any one of claims 74-78, wherein the end-processing enzyme comprises Trex2 or a biologically active fragment thereof.

80. The method of any one of claims 67-78, wherein the CAR T cells bound to a target antigen secrete an increased amount of one or more proinflammatory cytokines selected from the group consisting of: IFN γ , IL-4, IL-10, TNF α , IL-8, IL-5, IL-6, GM-CSF, and MIP-1 α , compared to a CAR T cell bound to the target antigen that lacks a modified TCR α allele.

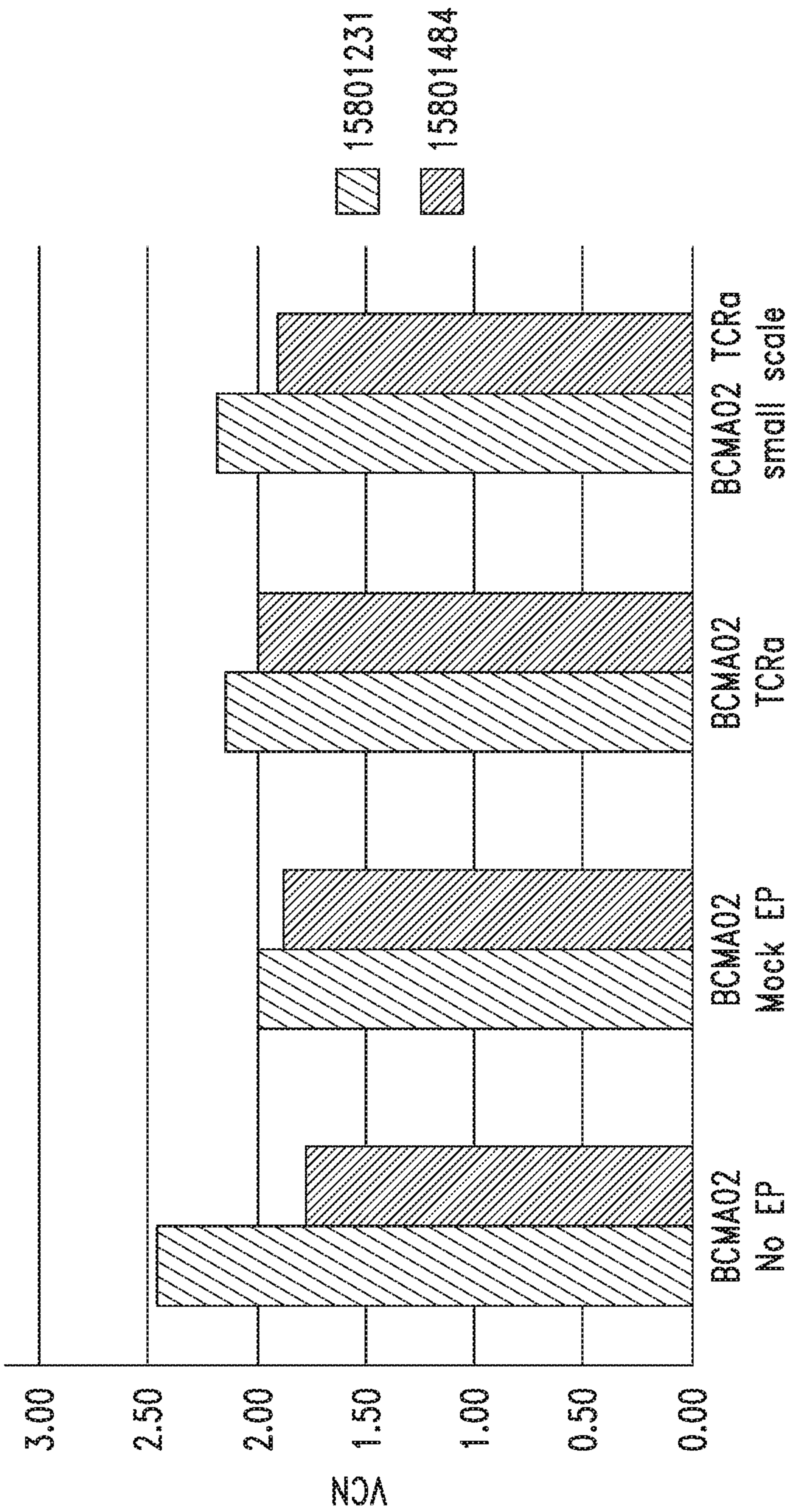


FIG. 1

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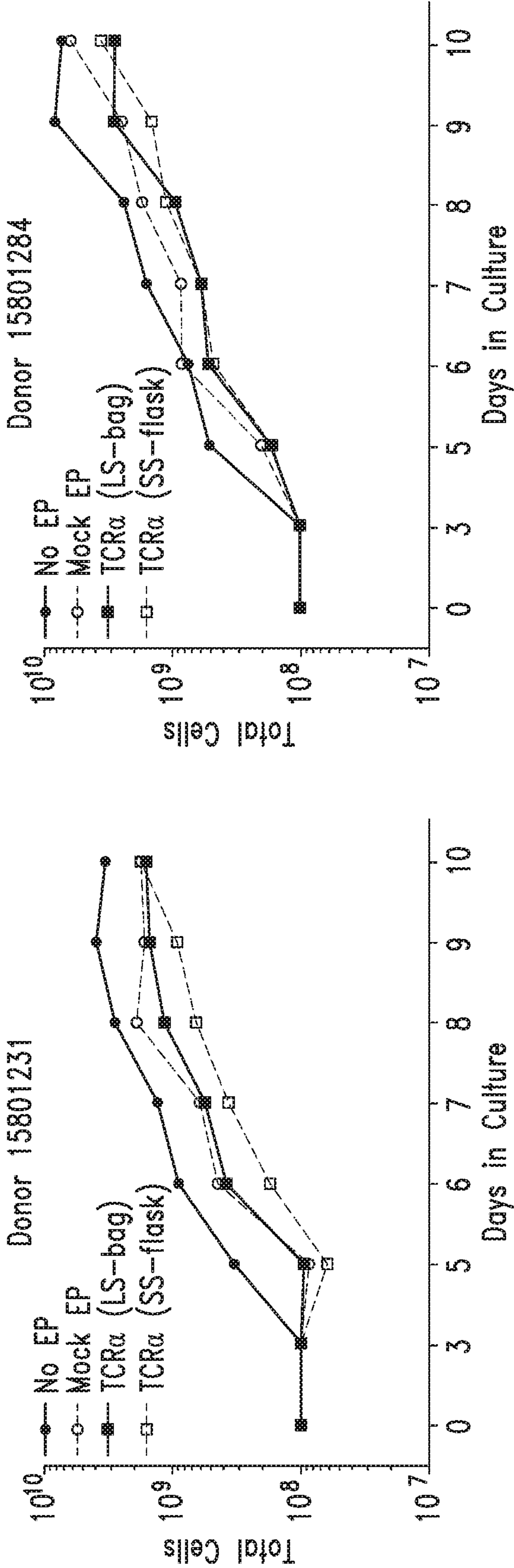
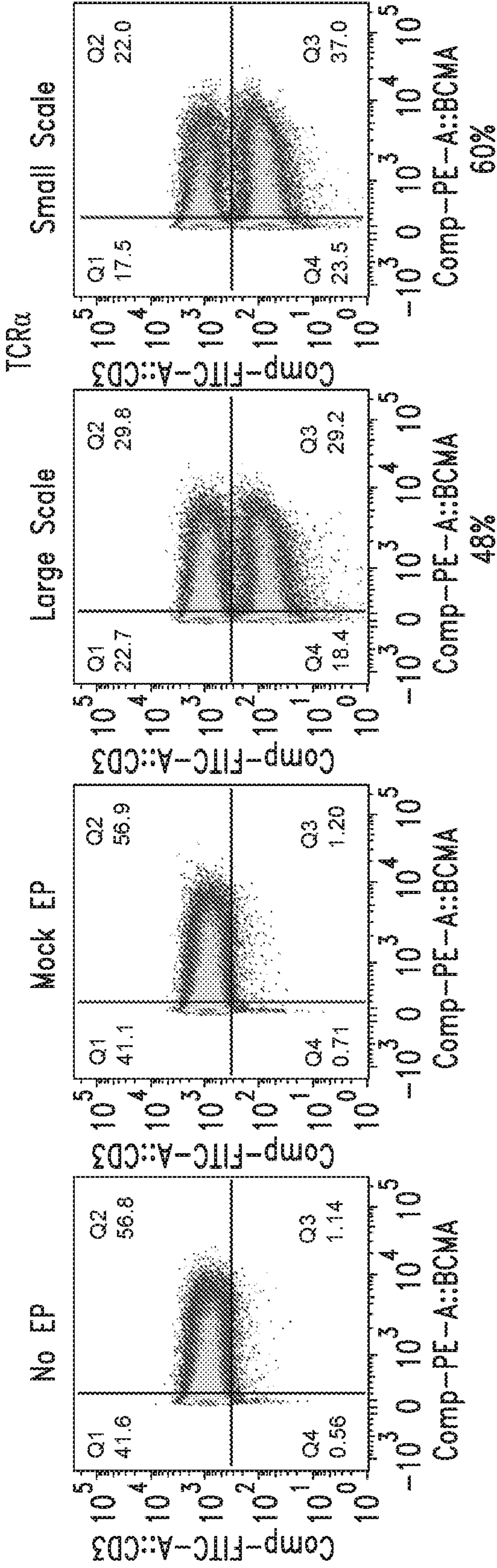
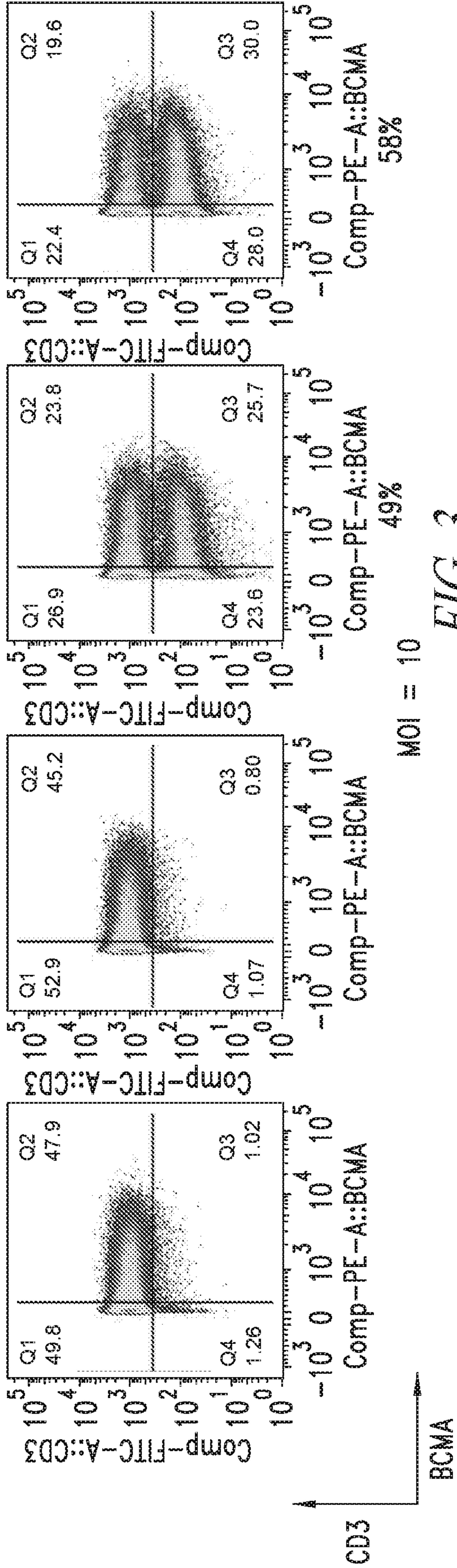


FIG. 2

Donor 15801231



Donor 15801284



MOI = 10

FIG. 3

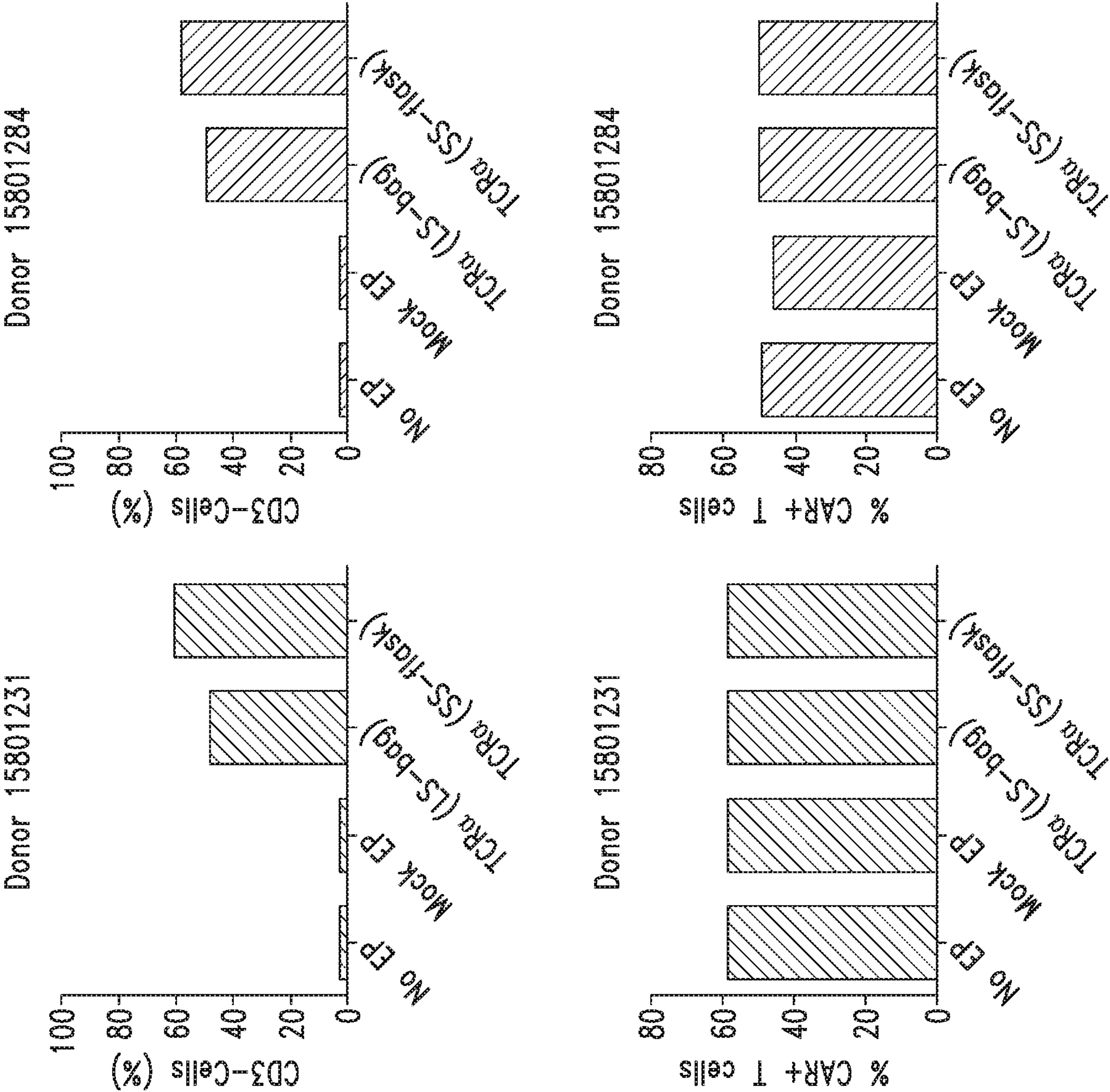


FIG. 4

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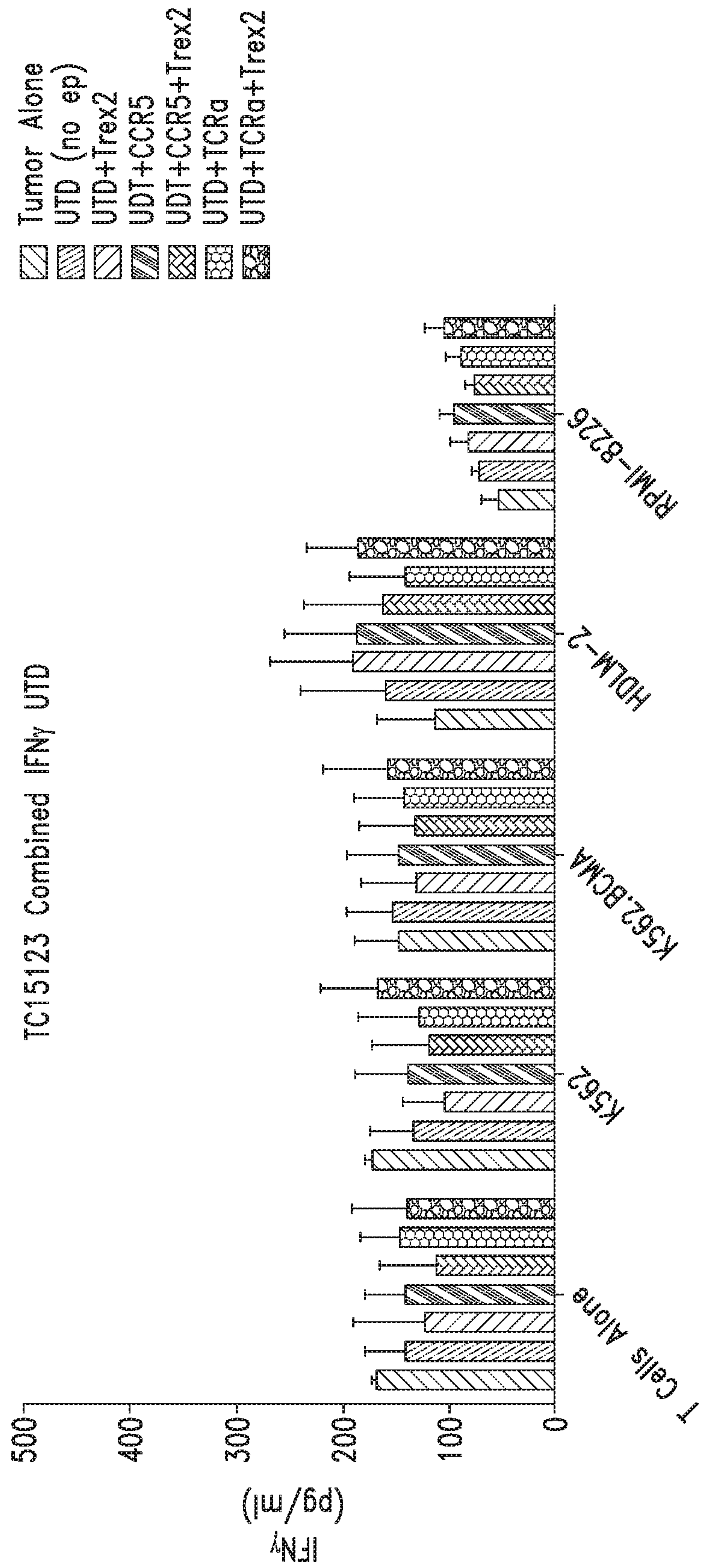


FIG. 5A

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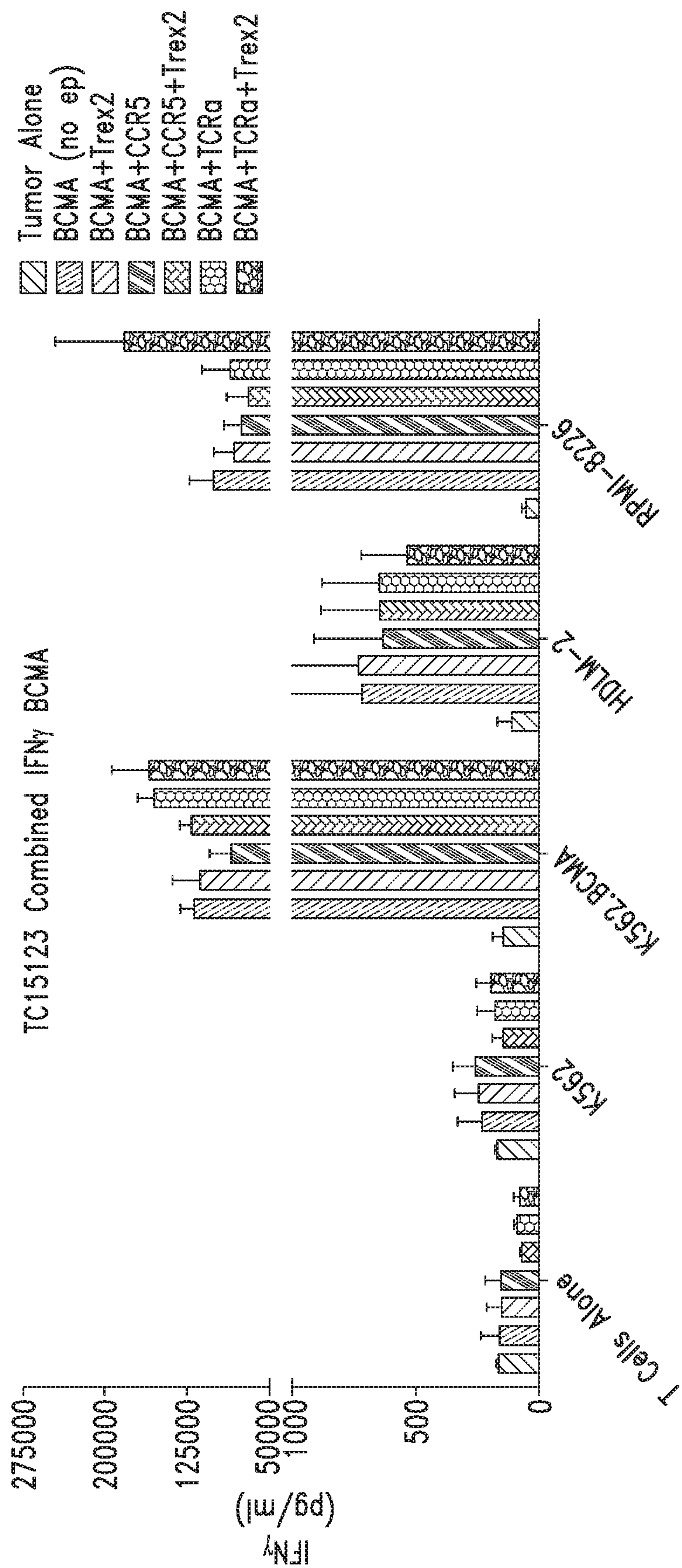


FIG. 5A (Continued)

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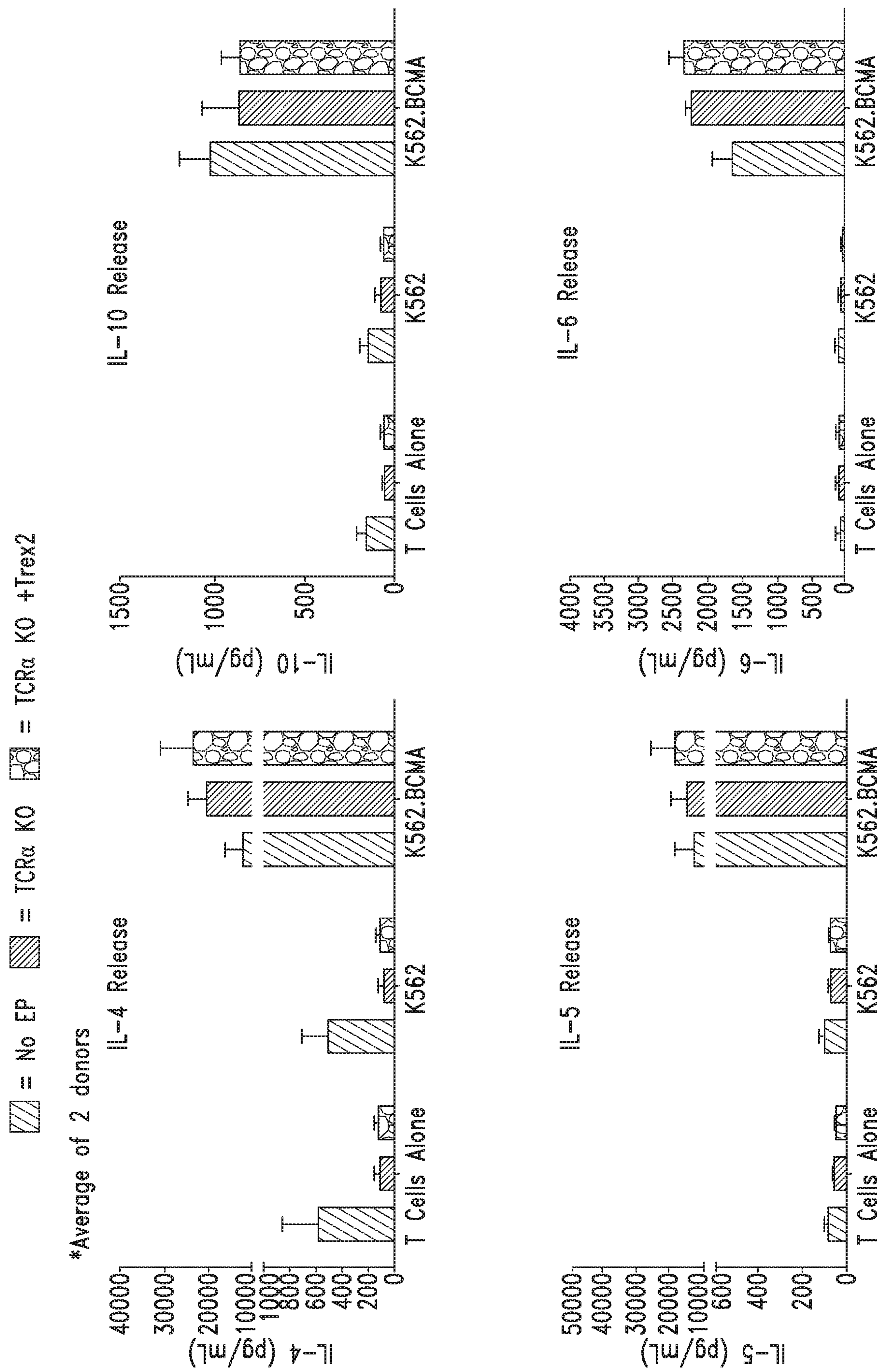


FIG. 5B

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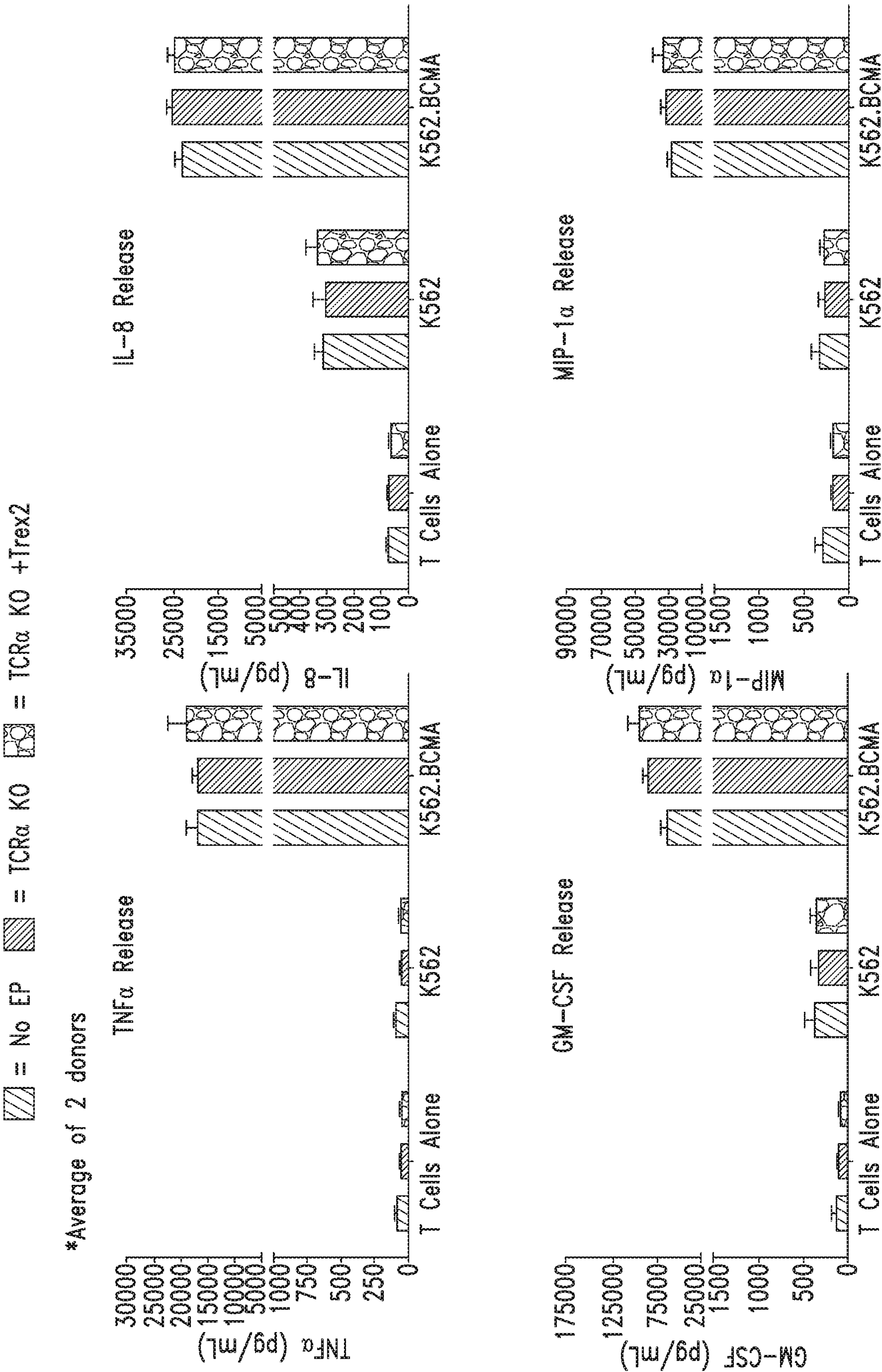
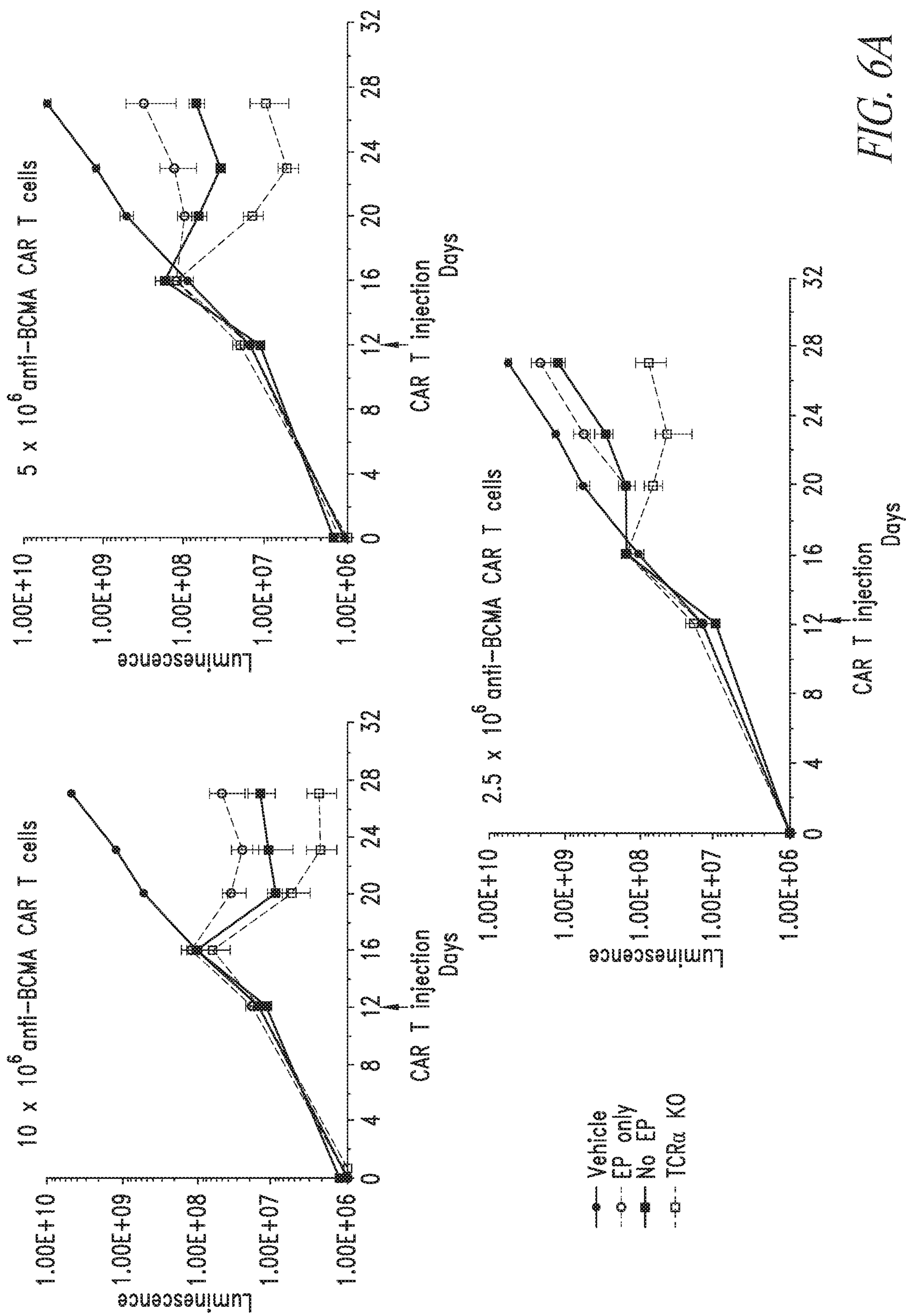


FIG. 5B (Continued)

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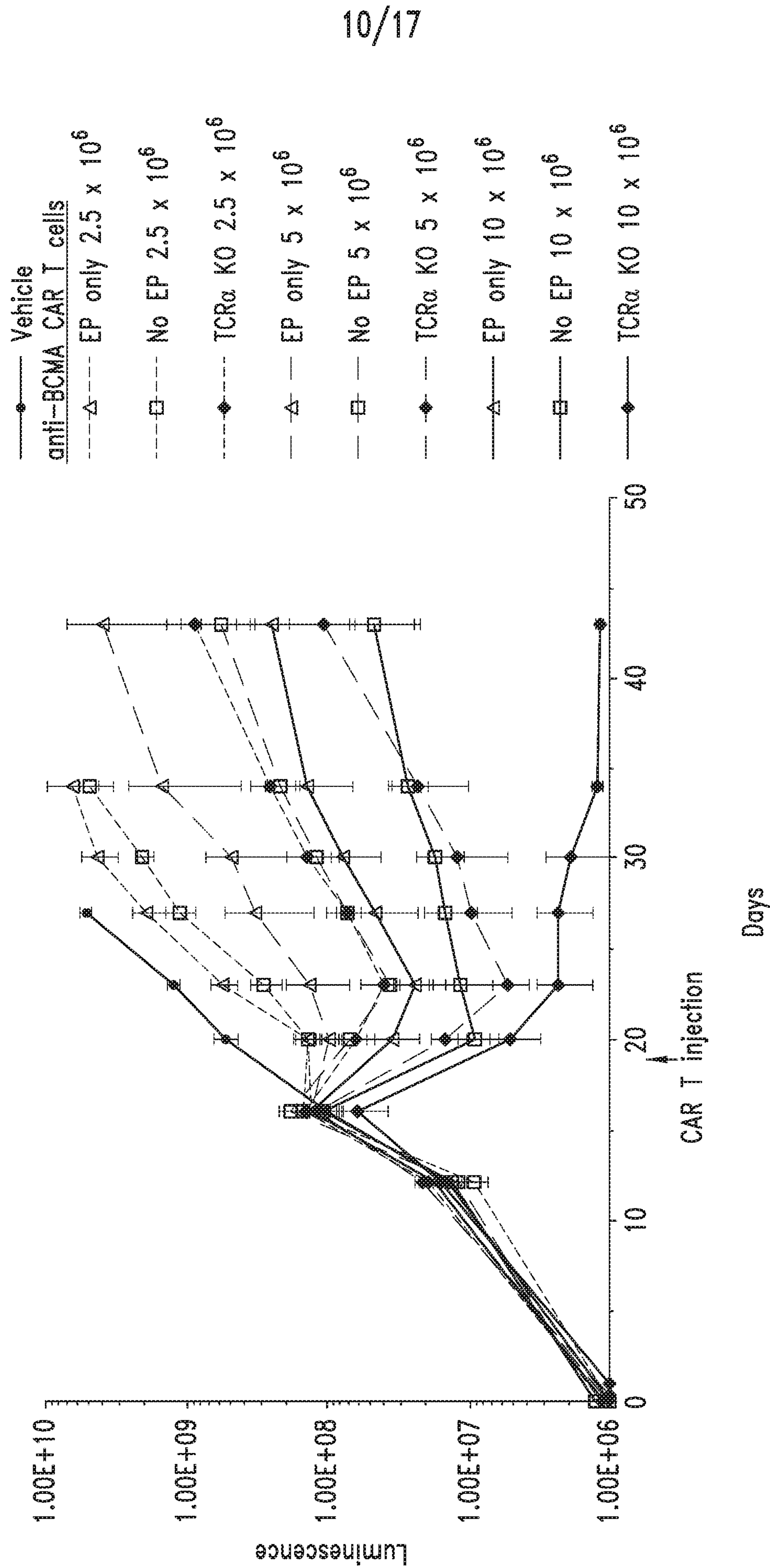
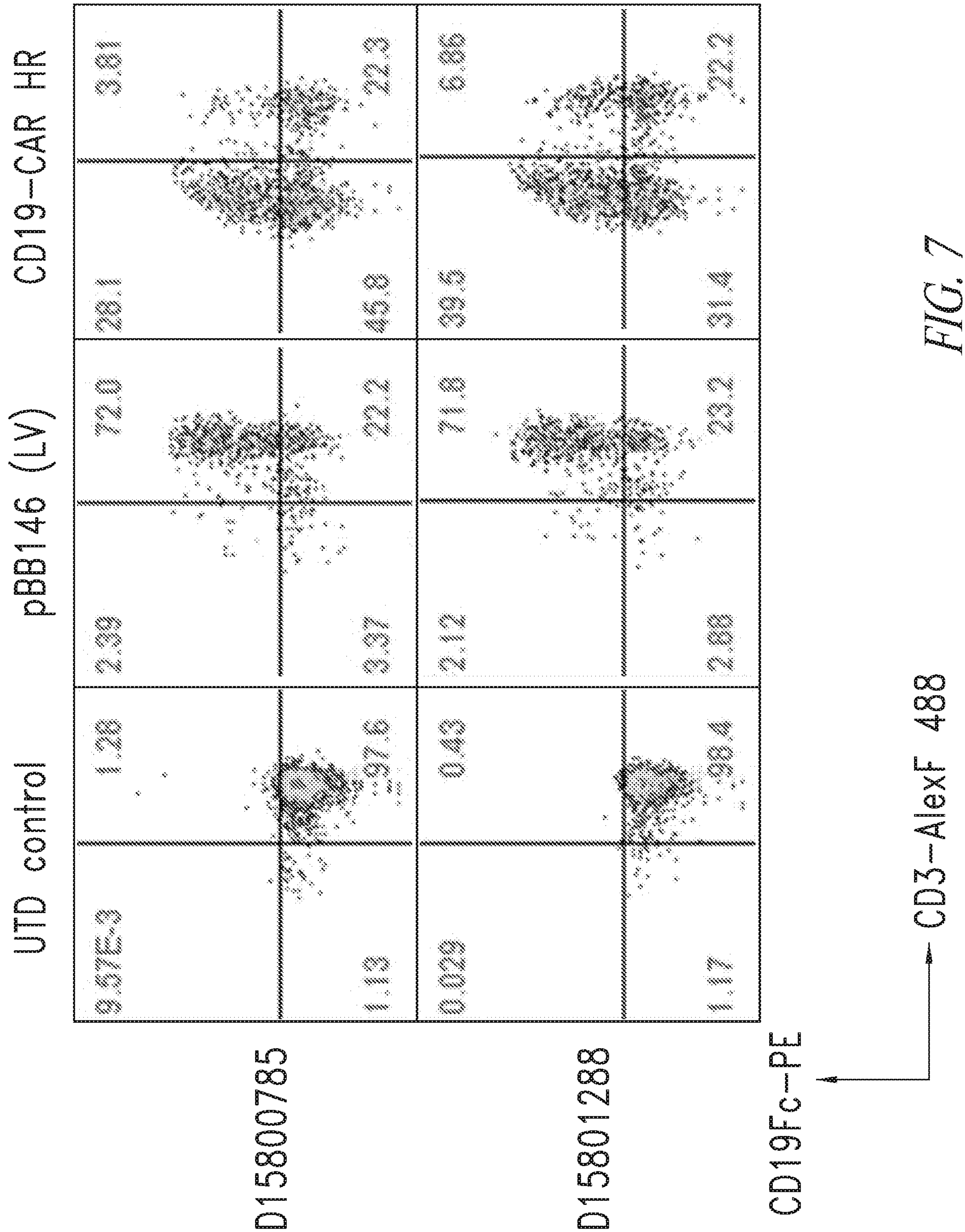


FIG. 6B



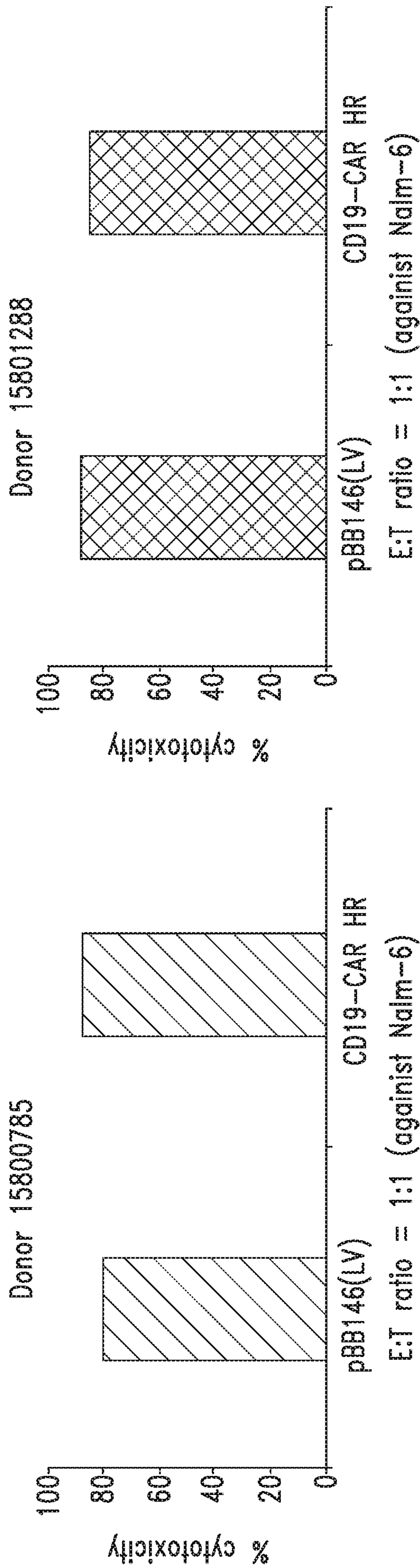


FIG. 8A

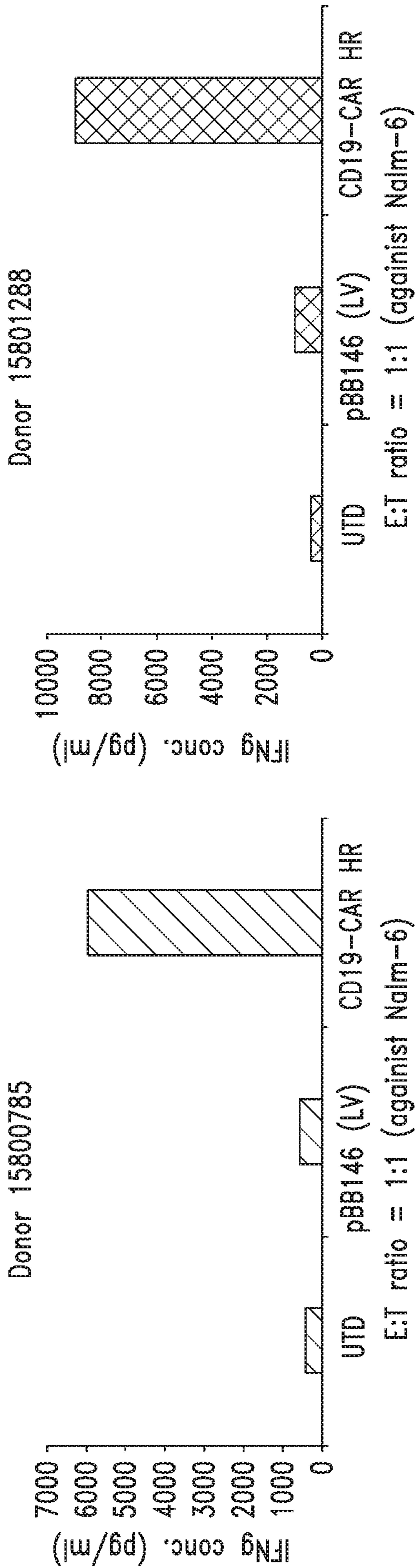


FIG. 8B

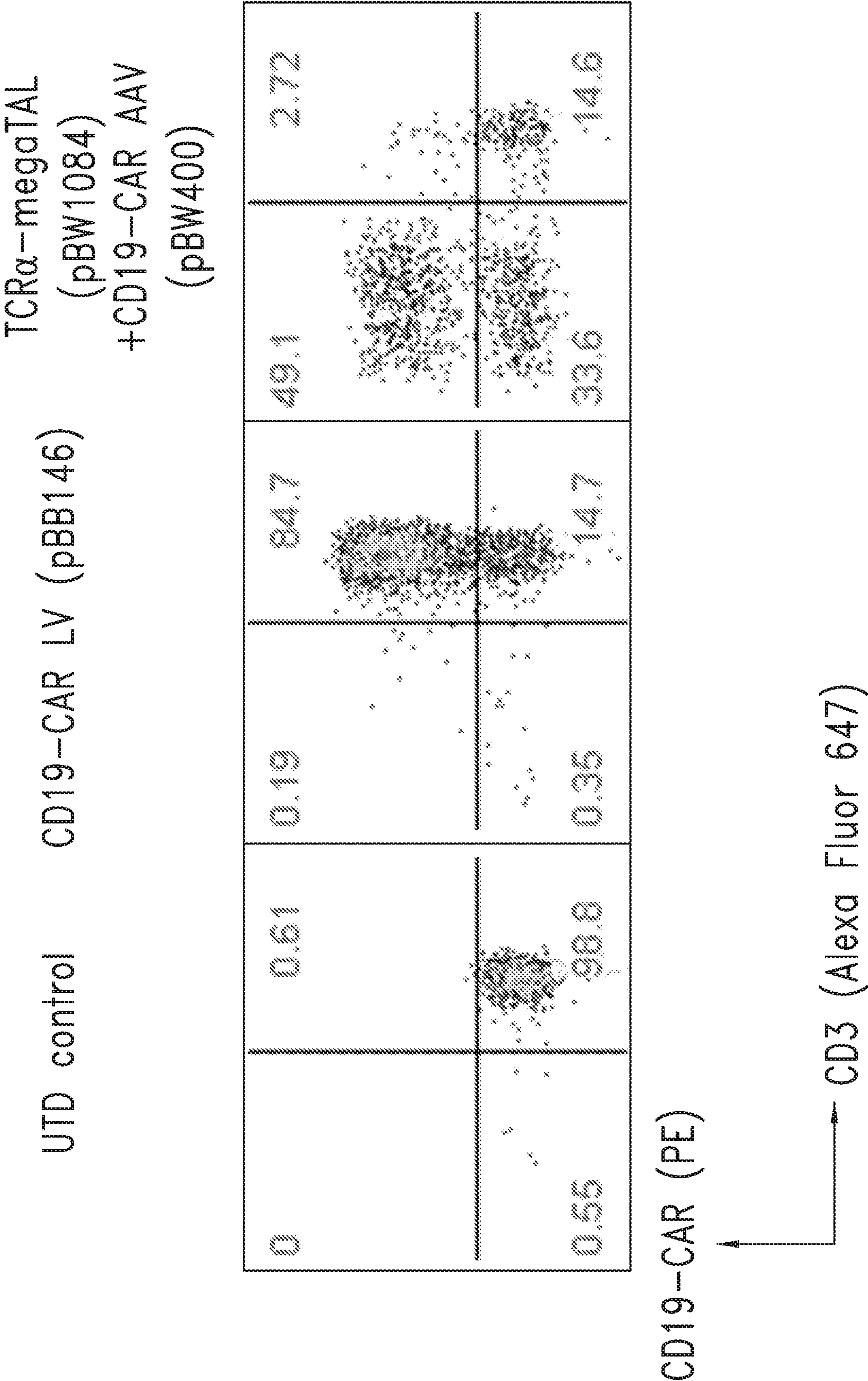


FIG. 9

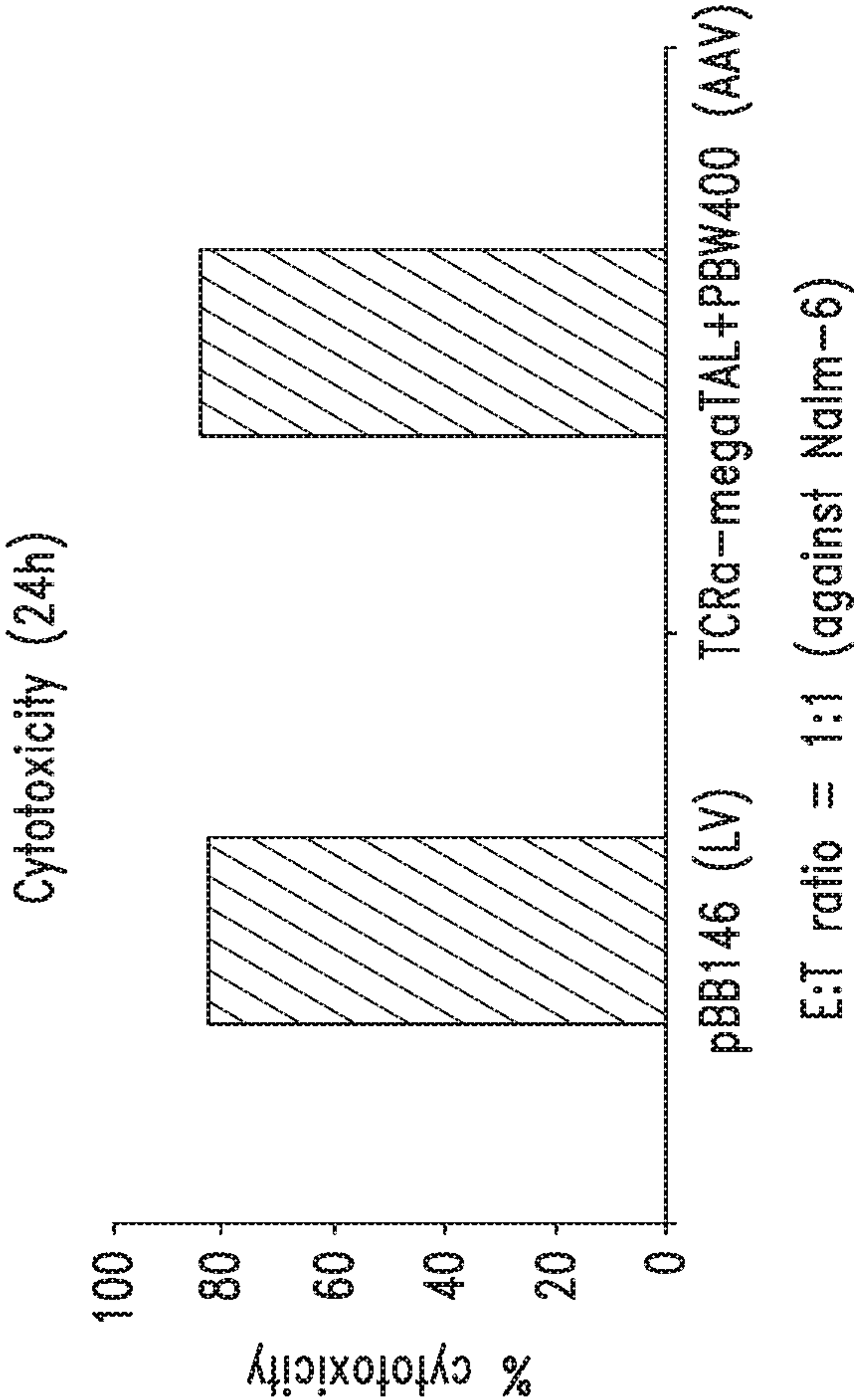


FIG. 10A

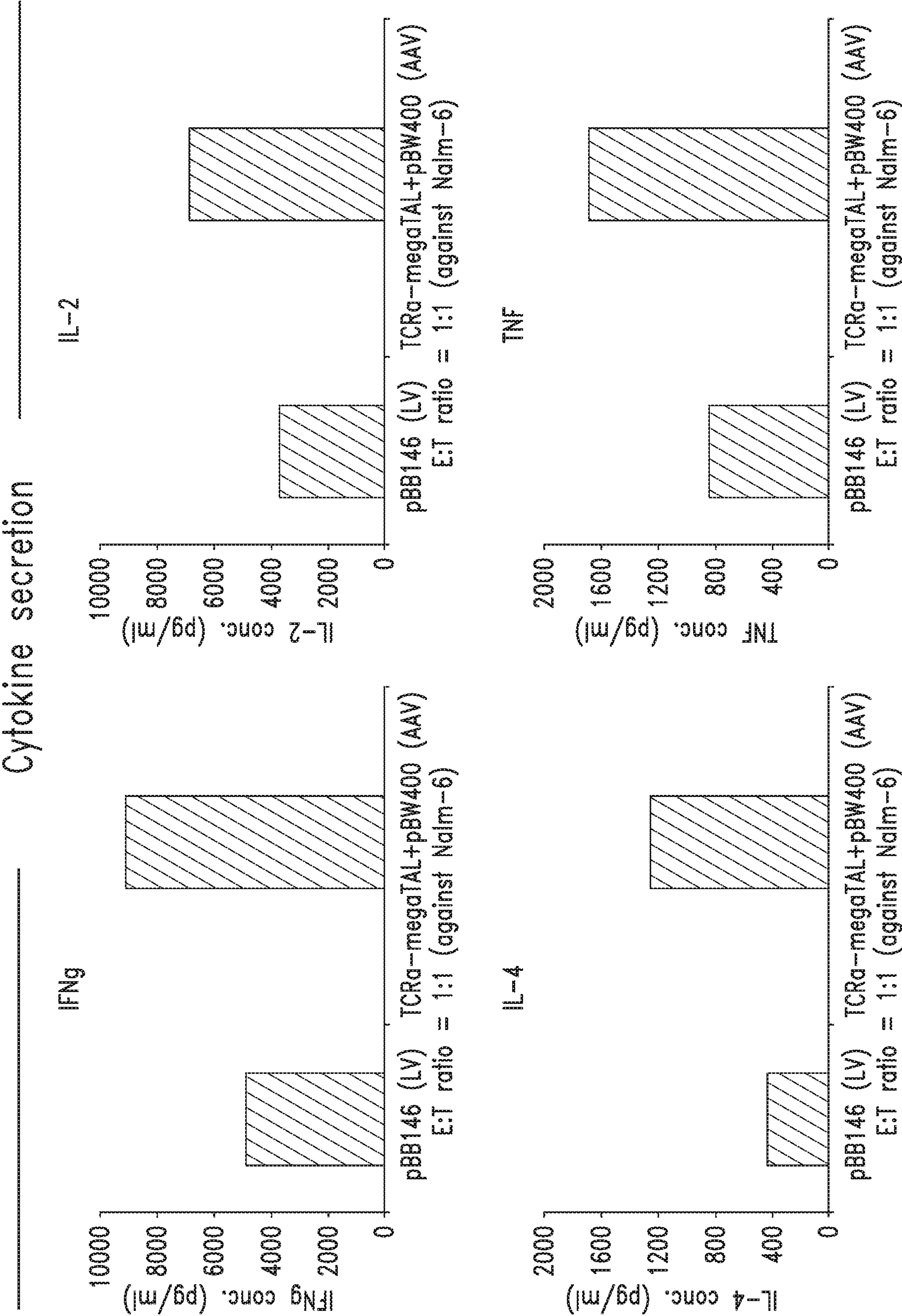
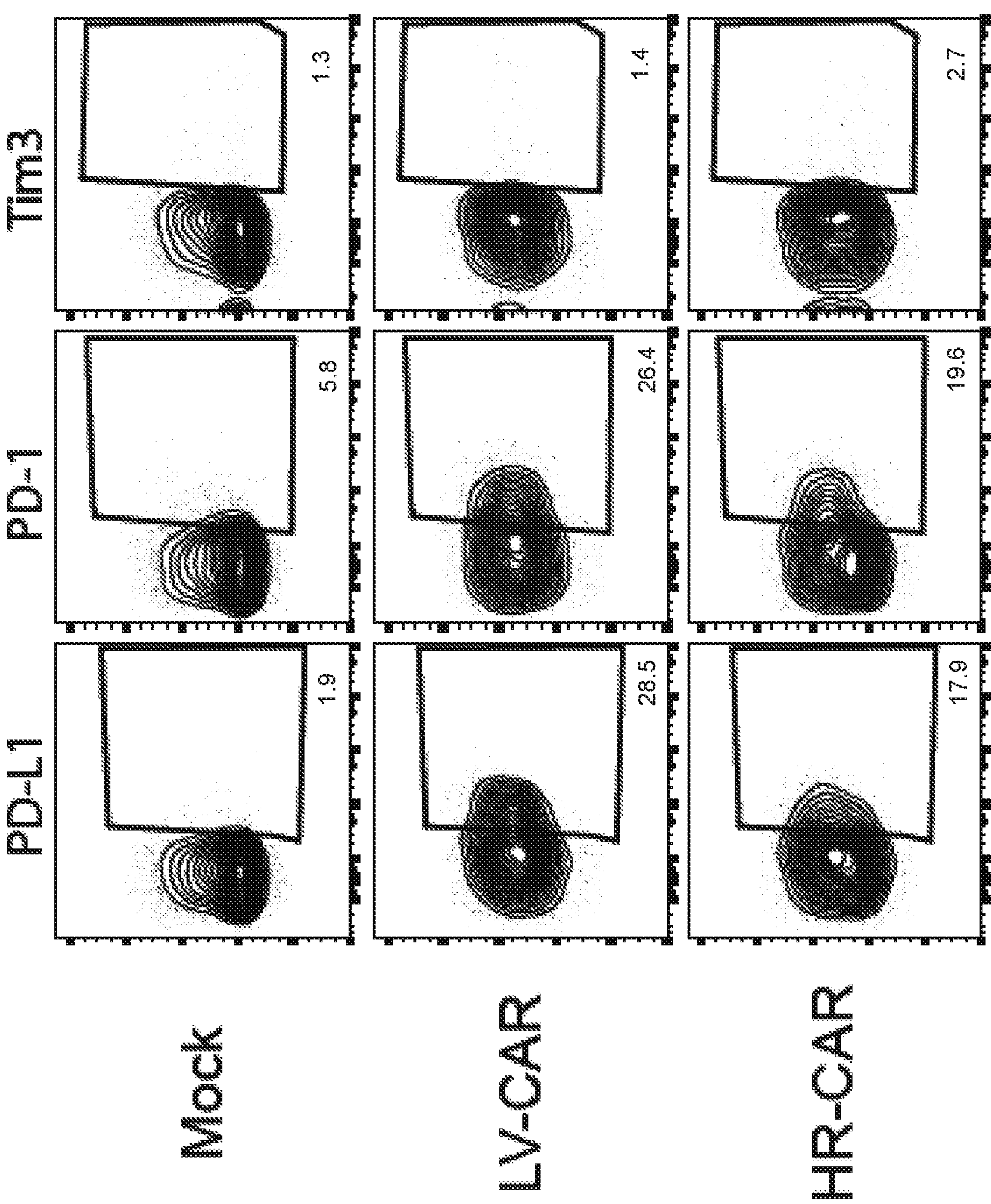
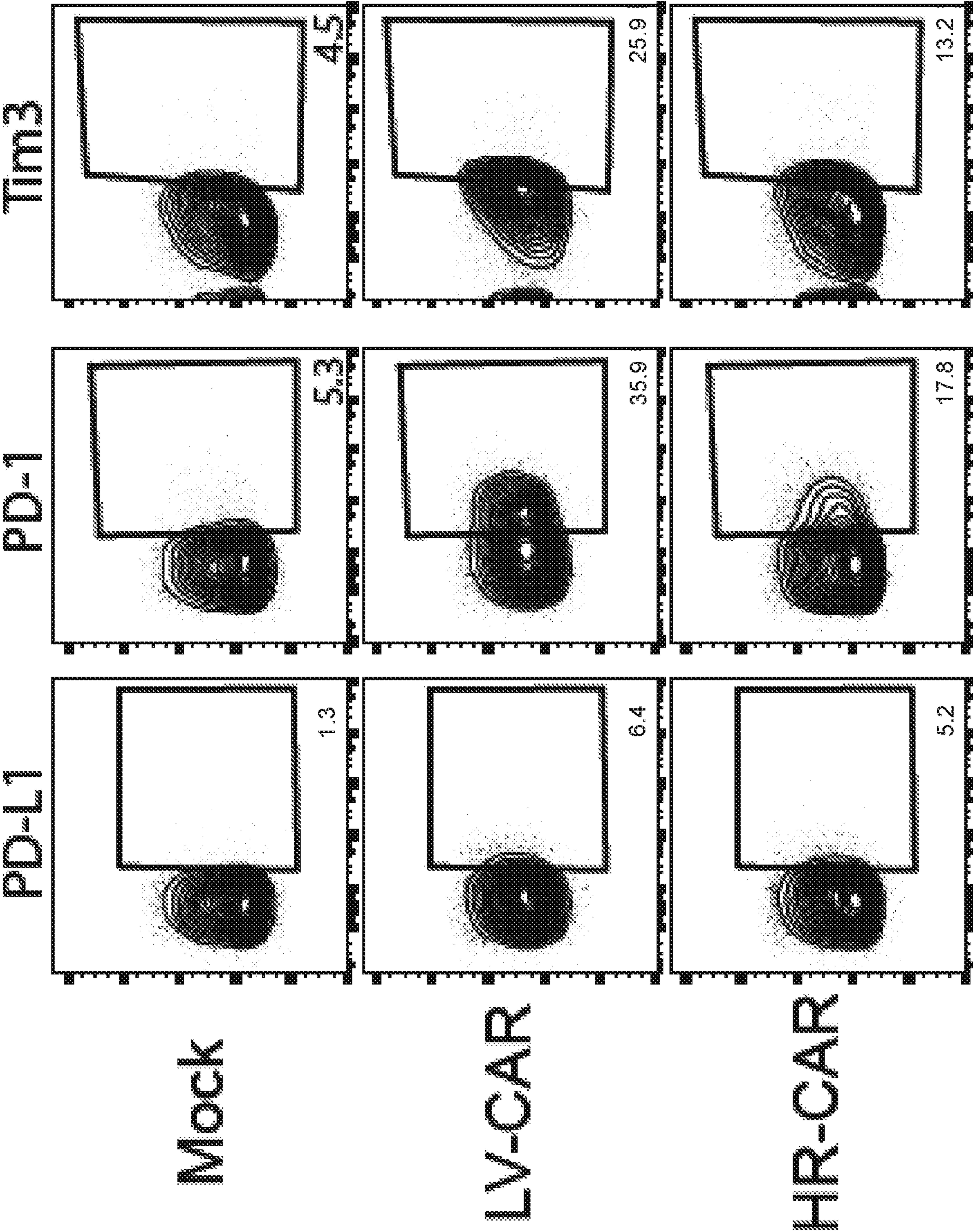


FIG. 10B



E:T = 1:1; Nalm6; 24hr co-culture
FIG. 11A



E:T = 1:1; Nalm6; 72hr co-culture

FIG. 11B