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METHODS AND COMPOSITIONS FOR TREATING CANCER

Abstract

Provided herein, in some embodiments, are methods and compositions (e.g., cell compositions) for the treatment of cancer.

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Background/Summary

RELATED APPLICATIONS [0001] The present application is a continuation of U.S. patent application Ser. No. 17/054,483, filed Nov. 10, 2020, which is a United States National Phase under 35 U.S.C. § 371 of International Application No. PCT/IB2019/000500, filed on May 10, 2019, which claims the benefit of U.S. Provisional Patent Application Ser. No. 62/670,417 filed May 11, 2018; U.S. Provisional Patent Application Ser. No. 62/701,340 filed Jul. 20, 2018; U.S. Provisional Patent Application Ser. No. 62/756,643 filed Nov. 7, 2018; U.S. Provisional Patent Application Ser. No. 62/773,658 filed Nov. 30, 2018; and U.S. Provisional Patent Application Ser. No. 62/826,600 filed Mar. 29, 2019. The entire contents of the above-referenced patent applications are incorporated by reference herein.

SEQUENCE LISTING
[0002] The application contains a Sequence Listing that has been filed electronically, created Jan. 13, 2025, and named "CRISP_42669_310_SequenceListing.xml", having a file size of 231,024 bytes, the contents of which are incorporated by reference herein in their entirety.
BACKGROUND
[0003] Chimeric antigen receptor (CAR) T-cell therapy uses genetically-modified T cells to more specifically and efficiently target and kill cancer cells. After T cells have been collected from the blood, the cells are engineered to include CARs on their surface. The CARs may be introduced into the T cells using CRISPR/Cas9 gene editing technology. When these allogeneic CAR T cells are injected into a patient, the receptors enable the T cells to kill cancer cells.
SUMMARY
[0004] In some aspects, the present disclosure provides engineered immune cells (e.g., T cells) and methods of producing immune cells that have been edited using CRISPR/Cas9 gene editing technology to disrupt endogenous CD70 expression (knockout CD70).
[0005] In some aspects of the present disclosure provide an engineered immune cell (e.g., T cell) comprising a disruption in the CD70 gene. In some embodiments, the engineered immune cells are allogeneic T cells comprising a disrupted CD70 gene and a nucleic acid encoding a CAR. In some embodiments, the engineered immune cells are allogeneic T cells comprising a TRAC gene disrupted by insertion of a nucleic acid encoding a CAR, a disrupted β2M gene, and a disrupted

CD70 gene. In some embodiments, the T cells are human T cells. In some embodiments, the engineered immune cells (e.g., T cells) comprise a disrupted TRAC gene, a disrupted B2M gene, a disrupted CD70 gene, and a nucleic acid encoding a CAR. In some embodiments, the disrupted TRAC gene comprises the nucleic acid encoding the CAR. In some embodiments the engineered immune cell (e.g., T cell) further comprises a disrupted PD-1 gene. In some embodiments the nucleic acid encoding a CAR target a tumor antigen (e.g., BCMA, CD19, CD33 or CD70).

[0006] In some aspects the engineered immune cell (e.g., T cell) provided exhibits improved T cell function including the prevention of premature exhaustion, enhanced CAR T cell expansion, and increased efficiency of cancer cell killing. In some aspects the engineered immune cell (e.g., T cell) provided exhibit continued, steady cell growth, relative to unedited T cells or relative to edited T cells that express CD70, as well as showing increased cytotoxicity and cytokine (e.g., IL-2 and/or IFN-gamma) secretion.

[0007] In some aspects, the disclosure provides an engineered T cell comprising a disrupted CD70 gene and a nucleic acid encoding a CAR that does not bind CD70. In some aspects, the engineered T cell comprises a disrupted T cell receptor alpha constant region (TRAC) gene. In some aspects, the disrupted TRAC gene comprises the nucleic acid encoding the CAR that does not bind CD70. In some aspects, the engineered T cell comprises a disrupted beta-2-microglobulin (β2M) gene.

[0008] In some aspects, the disclosure provides an engineered T cell comprising: (i) a disrupted TRAC gene; (ii) a disrupted B2M gene; (iii) a disrupted CD70 gene; and (iv) a nucleic acid encoding a CAR that does not bind CD70.

[0009] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise a disrupted CD70 gene and a nucleic acid encoding a CAR that does not bind CD70.

[0010] In some aspects, the engineered T cell in the population of cells comprises a disrupted T cell receptor alpha constant region (TRAC) gene. In some aspects, the disrupted TRAC gene comprises the nucleic acid encoding the CAR that does not bind CD70. In some aspects, the engineered T cell in the population of cells comprises a disrupted beta-2-microglobulin (β2M) gene.

[0011] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: (i) a disrupted TRAC gene; (ii) a disrupted B2M gene; (iii) a disrupted CD70 gene; and (iv) a nucleic acid encoding a CAR that does not bind CD70.

[0012] In any of the foregoing or related aspects, the CAR comprises an ectodomain that binds i-B cell maturation antigen (BCMA). In some aspects, the ectodomain comprises an anti-BCMA antibody. In some aspects, the ectodomain comprises an anti-BCMA single-chain variable fragment (scFv). In some aspects, the anti-BCMA scFv comprises variable heavy (VH) chain complementarity determining regions (CDRs) and the same variable light (VL) chain CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61. In some aspects, the anti-BCMA scFv comprises VH and VL chains comprising the amino acid sequences set forth in SEQ ID NOs: 60 and 61, respectively. In some aspects, the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59. In some aspects, the anti-BCMA scFv is encoded by a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID NO: 57.

[0013] In any of the foregoing or related aspects, the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59. In some embodiments, the anti-BCMA scFv comprises a VH comprising the amino acid sequence of SEQ ID NO: 60. In some embodiments, the anti-BCMA scFv comprises a VL comprising the amino acid sequence of SEQ ID NO: 61. In some embodiments, the anti-BCMA scFv comprises a VH comprising CDR amino acid sequences of (i) SEQ ID NO: 80, SEQ ID NO: 82, and/or SEQ ID NO: 84 or (ii) SEQ ID NO: 81, SEQ ID NO: 83, or SEQ ID NO: 85; and/or the anti-BCMA scFv comprises a VL sequence comprising CDR amino acid sequences of (i) SEQ ID NO: 74, SEQ ID NO: 76, and/or SEQ ID NO: 78.

[0014] In any of the foregoing or related aspects, the CAR comprises an ectodomain that binds

CD33. In some the ectodomain comprises an anti-CD33 antibody. In some aspects, the ectodomain comprises an anti-CD33 scFv. In some aspects, the anti-CD33 scFv comprises the same VH CDRs and the same VL chain CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 140 and a VL set forth as SEQ ID NO: 141. In some aspects, the anti-CD33 scFv comprises VH and VL chains comprising the amino acid sequences set forth in SEQ ID NOs: 140 and 141, respectively. In some aspects, the anti-CD33 scFv comprises the amino acid sequence of SEQ ID NO: 137.

[0015] In any of the foregoing or related aspects, the CAR comprises an ectodomain that binds CD19. In some aspects, wherein the ectodomain comprises an anti-CD19 antibody. In some aspects, the ectodomain comprises an anti-CD19 scFv. In some aspects, the anti-CD19 scFv comprises the same VH CDRs and the same VL chain CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 152 and a VL set forth as SEQ ID NO: 153. In some aspects, the anti-CD19 scFv comprises VH and VL chains comprising the amino acid sequences set forth in SEQ ID NOs: 152 and 153, respectively. In some aspects, the anti-CD19 scFv comprises the amino acid sequence of SEQ ID NO: 151.

[0016] In some aspects, the disclosure provides an engineered T cell comprising: (i) a disrupted TRAC gene; (ii) a disrupted B2M gene; (iii) a disrupted CD70 gene; and (iv) a nucleic acid encoding a CAR that binds CD70. In some aspects, the disrupted TRAC gene comprises the nucleic acid encoding the CAR.

[0017] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: (i) a disrupted TRAC gene; (ii) a disrupted B2M gene; (iii) a disrupted CD70 gene; and (iv) a nucleic acid encoding a CAR that binds CD70.

[0018] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0019] (i) a disrupted TRAC gene; [0020] (ii) a disrupted β2M gene; [0021] (iii) a disrupted CD70 gene [0022] (iv) a nucleic acid encoding a CAR comprising (a) an ectodomain that comprises an anti-CD70 scFv, (b) a CD8 transmembrane domain, and (c) an endodomain that comprises a 41BB co-stimulatory domain and a CD3z signaling domain.

[0023] In any of the foregoing or related aspects, the CAR that binds CD70 comprises an ectodomain comprising an anti-CD70 antibody. In some aspects, CAR comprises an ectodomain comprising an anti-CD70 scFv. In some aspects, the anti-CD70 scFv comprises the same VH CDRs and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 51 and a VL set forth as SEQ ID NO: 52. In some aspects, the anti-CD70 scFv comprises VH and VL chains comprising the amino acid sequences set forth in SEQ ID NOs: 51 and 52, respectively. In some aspects, the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 48 or 50. In some aspects, the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 50.

[0024] In any of the foregoing or related aspects, the anti-CD70 scFv comprises a VH comprising the amino acid sequence of SEQ ID NO: 51. In some embodiments, the anti-CD70 scFv comprises a VL comprising the amino acid sequence of SEQ ID NO: 52. In some embodiments, the anti-CD70 scFv comprises a VH comprising CDR amino acid sequences of (i) SEQ ID NO: 68, SEQ ID NO: 70, and/or SEQ ID NO: 72 or (ii) SEQ ID NO: 69, SEQ ID NO: 71, and/or SEQ ID NO: 73; and/or the anti-CD70 scFv comprises a VL sequence comprising CDR amino acid sequences of (i) SEQ ID NO: 62, SEQ ID NO: 64, and/or SEQ ID NO: 66 or (ii) SEQ ID NO: SEQ ID NO: 63, SEQ ID NO: 65, and/or SEQ ID NO: 67.

[0025] In any of the foregoing or related aspects, the CAR comprises a CD28 or 41BB co-stimulatory domain. In any of the foregoing or related aspects, the CAR comprises a CD3ζ signaling domain. In any of the foregoing or related aspects, the CAR comprises a CD8 transmembrane domain.

[0026] In any of the foregoing or related aspects, there is a deletion in the TRAC gene relative to

unmodified T cells. In some aspects, the deletion is 15-30 base pairs. In some aspects, the deletion is 20 base pairs. In some aspects, the deletion comprises SEQ ID NO: 86. In some aspects, the deletion is of SEQ ID NO: 86.

[0027] In some aspects, the disclosure provides an engineered T cell comprising a disrupted CD70 gene and a nucleic acid encoding a CAR that binds CD70, wherein the CAR comprises the amino acid sequence set forth in SEQ ID NO: 46. In some aspects, the disclosure provides an engineered T cell comprising a disrupted CD70 gene, and a nucleic acid encoding a CAR that binds CD70, wherein the nucleic acid sequence is at least 90% identical to SEQ ID NO: 45. In some aspects, the disclosure provides an engineered T cell comprising a disrupted CD70 gene, and a nucleic acid encoding a CAR that binds CD70, wherein the nucleic acid sequence is SEQ ID NO: 45.

[0028] In some embodiments, the CD70 gene is disrupted by CRISPR/Cas9 gene editing. In some embodiments, the TRAC gene is disrupted by CRISPR/Cas9 gene editing. In some embodiments, the B2M gene is disrupted by CRISPR/Cas9 gene editing. In some embodiments, the PD-1 gene is disrupted by CRISPR/Cas9 gene editing.

[0029] In some aspects, the disclosure provides an engineered T cell comprising: [0030] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR comprising the amino acid sequence set forth in SEQ ID NO: 46; [0031] (ii) a disrupted B2M gene; and [0032] (iii) a disrupted CD70 gene. In some embodiments, the nucleic acid encoding the CAR comprises a sequence at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 45.

[0033] In other aspects, the disclosure provides an engineered T cell comprising: [0034] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR, wherein the nucleic acid sequence is at least 90% identical to SEQ ID NO: 45; [0035] (ii) a disrupted B2M gene; and [0036] (iii) a disrupted CD70 gene. In some embodiments, the disrupted TRAC gene comprises a donor sequence comprising the nucleotide sequence set forth in SEQ ID NO: 45 or SEQ ID NO: 44.

[0037] In some aspects, the disclosure provides an engineered T cell comprising: [0038] (i) a disrupted TRAC gene comprising a nucleic acid sequence at least 90% identical to SEQ ID NO: 44; [0039] (ii) a disrupted B2M gene; and [0040] (iii) a disrupted CD70 gene.

[0041] In some aspects, the disclosure provides an engineered T cell comprising: [0042] (i) a disrupted TRAC gene comprising the nucleic acid sequence of SEQ ID NO: 44; [0043] (ii) a disrupted B2M gene; and [0044] (iii) a disrupted CD70 gene.

[0045] In any of the foregoing or related aspects, the engineered T cell comprises a disrupted PD-1 gene. In some aspects, the engineered immune cells are allogeneic T cells comprising a TRAC gene disrupted by insertion of a nucleic acid encoding a CAR, a disrupted β2M gene, and a disrupted PD-1 gene. In some embodiments the engineered immune cell (e.g., T cell) further comprises a disrupted CD70 gene.

[0046] In any of the foregoing or related aspects, the engineered T cell maintains cytotoxicity following 5 rechallenges with a target cell, wherein the target cell expresses an antigen specific for the CAR. In some aspects, the engineered T cell maintains cytotoxicity following 10 rechallenges with the target cell. In some aspects, the target cell is a cancer cell. In some aspects, the target cell is a cancer cell of a hematological cancer or solid tumor.

[0047] In any of the foregoing or related aspects, the engineered T cell or population of cells comprises a CAR comprising the amino acid sequence of SEQ ID NO: 57. In some aspects, the CAR is encoded by a nucleic acid sequence having at least 90% identity to SEQ ID NO: 56.

[0048] In any of the foregoing or related aspects, the engineered T cell or population of cells comprises a CAR comprising the amino acid sequence of SEQ ID NO: 139. In some aspects, the CAR is encoded by a nucleic acid sequence having at least 90% identity to SEQ ID NO: 136.

[0049] In any of the foregoing or related aspects, the engineered T cell or population of cells comprises a CAR comprising the amino acid sequence of SEQ ID NO: 149. In some aspects, the

CAR is encoded by a nucleic acid sequence having at least 90% identity to SEQ ID NO: 148.

[0050] In any of the foregoing or related aspects, the engineered T cell or population of cells comprises a CAR comprising the amino acid sequence of SEQ ID NO: 46. In some aspects, the CAR is encoded by a nucleic acid sequence having at least 90% identity to SEQ ID NO: 45.

[0051] Other aspects of the present disclosure provide a population of cells comprising any of the engineered immune cells (e.g., T cells) described herein. In some embodiments, a population of cells comprise T cells that comprise a TRAC gene disrupted by insertion of a nucleic acid encoding a CAR, a disrupted β2M gene, and a disrupted CD70 gene. In some embodiments, a population of cells comprise T cells that comprise a disrupted TRAC gene, a disrupted B2M gene, a disrupted CD70 gene, and a nucleic acid encoding a CAR. In some embodiments, a population of cells comprise T cells that comprise a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR, a disrupted B2M gene, and a disrupted CD70 gene.

[0052] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0053] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR comprising (a) an ectodomain that comprises an anti-CD70 antigen-binding fragment, (b) a CD8 transmembrane domain, and (c) an endodomain that comprises a 41BB co-stimulatory domain and a CD3z signaling domain; [0054] (ii) a disrupted beta-2-microglobulin (B2M) gene; and [0055] (iii) a disrupted CD70 gene.

[0056] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0057] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR comprising the amino acid sequence set forth in SEQ ID NO: 46; [0058] (ii) a disrupted β2M gene; and [0059] (iii) a disrupted CD70 gene.

[0060] In other aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0061] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR, wherein the nucleic acid sequence is at least 90% identical to SEQ ID NO: 45; [0062] (ii) a disrupted β2M gene; and [0063] (iii) a disrupted CD70 gene. In some aspects, the disrupted TRAC gene comprises the nucleic acid sequence set forth in SEQ ID NO: 45.

[0064] In some aspects, the disclosure provides a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0065] (i) a disrupted TRAC gene comprising a nucleic acid sequence at least 90% identical to SEQ ID NO: 44; [0066] (ii) a disrupted β2M gene; and [0067] (iii) a disrupted CD70 gene. In some aspects, the disrupted TRAC gene comprises the nucleic acid sequence set forth in SEQ ID NO: 44.

[0068] In some embodiments, the CAR comprises a CD3z signaling domain. In some embodiments, the CAR comprises a CD8 transmembrane domain. In some embodiments, the CAR comprises a CD28 or 41BB co-stimulatory domain.

[0069] In any of the foregoing or related aspects of the population of cells, the disrupted β2M gene comprises at least one nucleotide sequence selected from any one of SEQ ID NOS: 9-14. In any of the foregoing or related aspects of the population of cells, the disrupted CD70 gene comprises at least one nucleotide sequence selected from any one of SEQ ID NOS: 129-134.

[0070] In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 45 and/or the nucleic acid encoding the anti-CD70 CAR comprises the nucleotide sequence of SEQ ID NO: 45. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 45. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 44. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 56 and/or the nucleic acid encoding the anti-BCMA CAR comprises the nucleotide sequence of SEQ ID NO: 56. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 56. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 55.

[0071] In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO:

156 and/or the nucleic acid encoding the anti-CD19 CAR comprises the nucleotide sequence of SEQ ID NO: 148. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 148. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 156. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 135 and/or the nucleic acid encoding the anti-CD33 CAR comprises the nucleotide sequence of SEQ ID NO: 136. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 136. In some embodiments, the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 135.

[0072] In any of the foregoing aspects, the engineered T cells: (a) exhibit increased cellular proliferative capacity; [0073] (b) exhibit increased cell lysis; [0074] (c) exhibit reduced cellular exhaustion; [0075] (d) maintain cytokine-dependent proliferation; [0076] (e) exhibit increased cytokine secretion; or [0077] (f) any combination of (a)-(e), [0078] relative to control T cells, wherein control T cells express endogenous CD70 protein.

[0079] In some embodiments, at least 50%, optionally 50%-65%, of the engineered T cells do not express a detectable level of TCR surface protein, do not express a detectable level of β2M surface protein, do not express a detectable level of CD70 surface protein, and/or express a detectable level of the CAR.

[0080] In some embodiments, at least 90%, optionally 90%-100%, of the engineered T cells do not express a detectable level of TCR surface protein. In some embodiments, greater than 99.5% of the engineered T cells do not express a detectable level of TCR surface protein.

[0081] In some embodiments, at least 60%, optionally 60%-75%, of the engineered immune cells (e.g., T cells) do not express a detectable level of (β2M surface protein.

[0082] In some embodiments, at least 80%, optionally 80%-100%, of the engineered immune cells (e.g., T cells) do not express a detectable level of CD70 surface protein.

[0083] In some embodiments, at least 80%, optionally 80%-95%, of the engineered immune cells (e.g., T cells) express a detectable level of the CAR (e.g., an anti-CD70 CAR or an anti-BCMA CAR).

[0084] In some embodiments, the engineered immune cells (e.g., T cells) further comprise a disrupted PD-1 gene.

[0085] In some embodiments, at least 50%, optionally 50%-70%, of the engineered T cells do not express a detectable level of TCR surface protein, do not express a detectable level of (β2M surface protein, do not express a detectable level of PD-1 surface protein, do not express a detectable level of CD70 surface protein, and/or express a detectable level of the CAR.

[0086] In some aspects, the disclosure provides a method for producing an engineered T cell, the method comprising: [0087] (a) delivering to a T cell [0088] an RNA-guided nuclease, [0089] a gRNA targeting a CD70 gene, and [0090] a vector comprising a donor template that comprises a nucleic acid encoding a CAR; and [0091] (b) producing an engineered T cell comprising a disrupted CD70 gene and expressing the CAR.

[0092] In some aspects, the method further comprises delivering to the T cell a gRNA targeting a TRAC gene; wherein the engineered T cell further comprises a disrupted TRAC gene. In some aspects, the nucleic acid encoding the CAR is flanked by left and right homology arms to the TRAC gene; and wherein the engineered T cell comprises the nucleic acid encoding the CAR in the TRAC gene. In some aspects, the method further comprises delivering to the T cell a gRNA targeting a β2M gene; wherein the engineered T cell of further comprises a disrupted β2M gene.

[0093] Also provided herein are methods for producing an engineered T cell, the method comprising (a) delivering to a T cell an RNA-guided nuclease, a gRNA targeting a TRAC gene, a gRNA targeting a β2M gene, a gRNA targeting a CD70 gene, and a vector comprising a donor template that comprises a nucleic acid encoding a CAR, optionally wherein the nucleic acid encoding the CAR is flanked by left and right homology arms to the TRAC gene locus, and (b) producing an engineered T cell.

[0094] In some embodiments, the RNA-guided nuclease is a Cas9 nuclease, optionally a *Streptococcus pyogenes* Cas9 nuclease. Other RNA-guided nucleases may be used and are described below.

[0095] In some embodiments, wherein the gRNA targeting the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 98 or targets the nucleotide sequence of SEQ ID NO: 118, and optionally wherein the gRNA targeting the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 30. In some embodiments, the gRNA targeting the β2M gene comprises the nucleotide sequence of SEQ ID NO: 99 or targets the nucleotide sequence of SEQ ID NO: 119, and optionally wherein the gRNA targeting the β2M gene comprises the nucleotide sequence of SEQ ID NO: 31. In some embodiments, the gRNA targeting the CD70 gene comprises the nucleotide sequence of SEQ ID NOS: 94 or 95 or targets the nucleotide sequence of SEQ ID NO: 114 or 115, and optionally wherein the gRNA targeting the CD70 gene comprises the nucleotide sequence of SEQ ID NOS: 26 or 27.

[0096] In any of the foregoing aspects, the RNA-guided nuclease and gRNA are complexed in a ribonucleorotein particle (RNP).

[0097] In some embodiments, the methods further comprise delivering to the T cell a gRNA targeting a PD-1 gene.

[0098] In some embodiments, the gRNA targeting the PD-1 gene comprises the nucleotide sequence of SEQ ID NO: 100 or targets the nucleotide sequence of SEQ ID NO: 120, and optionally wherein the gRNA targeting the PD-1 gene comprises the nucleotide sequence of SEQ ID NO: 32.

[0099] In some aspects, the disclosure provides a method for producing an engineered T cell for immunotherapy against a target cell, comprising: [0100] (a) disrupting a CD70 gene in a T cell, and [0101] (b) expressing a CAR that binds to an antigen expressed on the target cell, wherein the antigen is not CD70. In some aspects, the target cell is a cancer cell. In some aspects, the method is ex vivo. In some aspects, the method further comprises comprising disrupting a TRAC gene in the T cell. In some aspects, the method further comprises disrupting a β2M gene in the T cell. In some aspects, the CAR is encoded by a nucleic acid in the disrupted TRAC gene. In some aspects, the CAR is any one of the CARs described herein.

[0102] In some aspects, the disclosure provides a population of engineered T cells produced by any one of the methods described herein.

[0103] In some aspects, the disclosure provides a method of increasing proliferation of T cells, comprising disrupting the CD70 gene in the T cells. In some aspects, the disclosure provides a method of reducing exhaustion of T cells, comprising disrupting the CD70 gene in the T cells.

[0104] In any of the foregoing aspects, the CD70 gene is disrupted by CRISPR/Cas gene editing. In some aspects, the method further comprises disrupting the TRAC gene, the β2M gene, or both the TRAC and β2M genes in the T cells. In some aspects, the TRAC gene, β2M gene or both TRAC and β2M gene is disrupted by CRISPR/Cas gene editing.

[0105] In some embodiments, the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 46. In some embodiments, the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 57. In some embodiments, the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 149. In some embodiments, the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 139.

[0106] In some aspects, the disclosure provides methods for administering the population of cells or an engineered T cells described herein to a subject. In some aspects, the engineered T cells are engineered human T cells. In some aspects, the subject has cancer. In some aspects, the cancer expresses CD70, BMCA, CD19, CD33 or combinations thereof. In some aspects, the population of cells is administered to the subject in an amount effective to treat the cancer. In some aspects, the cancer is a solid tumor malignancy or a hematological malignancy. In some aspects, the solid tumor

malignancy is selected from the group consisting of: ovarian tumor, pancreatic tumor, kidney tumor, lung tumor, and intestinal tumor. In some aspects, the population of cells is administered to the subject in an amount effective to reduce the volume of a tumor in the subject.

[0107] In some aspects, the disclosure provides a method for treating cancer in a subject, comprising administering the population of cells or an engineered T cells described herein to a subject.

[0108] In some aspects, the disclosure provides a method for treating cancer in a subject, comprising administering to the patient a population of cells comprising engineered T cells, wherein the engineered T cells comprise a disrupted CD70 gene and a nucleic acid encoding a CAR, thereby treating cancer in the subject. In some embodiments, the CAR binds CD70. In some embodiments, the CAR does not bind CD70.

[0109] In other aspects, the disclosure provides a method for treating cancer in a subject, comprising administering to the patient a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0110] (i) a disrupted TRAC gene; [0111] (ii) a disrupted B2M gene; [0112] (iii) a disrupted CD70 gene; and [0113] (iv) a nucleic acid encoding a CAR; [0114] thereby treating the cancer in the subject.

[0115] In yet other aspects, the disclosure provides a method for treating cancer in a subject, comprising administering to the patient a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0116] (i) a disrupted TRAC gene; [0117] (ii) a disrupted B2M gene; [0118] (iii) a disrupted CD70 gene; and [0119] (iv) a nucleic acid encoding a CAR comprising (a) an ectodomain that comprises an anti-CD70 antigen-binding fragment, (b) a CD8 transmembrane domain, and (c) an endodomain that comprises a 41BB co-stimulatory domain and a CD3z signaling domain, [0120] thereby treating the cancer in the subject. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 46. In some embodiments, the nucleic acid encoding the CAR comprises the nucleotide sequence of SEQ ID NO: 45. In some embodiments, the disrupted TRAC gene comprises the nucleotide sequence of SEQ ID NO: 45 or SEQ ID NO: 44.

[0121] In some aspects, the disclosure provides a method of treating cancer in a subject, comprising administering to the subject a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0122] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR comprising the amino acid sequence set forth in SEQ ID NO: 46; [0123] (ii) a disrupted β2M gene; and [0124] (iii) a disrupted CD70 gene, [0125] thereby treating the cancer in the subject.

[0126] In some aspects, the disclosure provides a method of treating cancer in a subject, comprising administering to the subject a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0127] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR, wherein the nucleic acid sequence is at least 90% identical to SEQ ID NO: 45; [0128] (ii) a disrupted β2M gene; and [0129] (iii) a disrupted CD70 gene, [0130] thereby treating the cancer in the subject. In some aspects, the disrupted TRAC gene comprises the nucleic acid sequence set forth in SEQ ID NO: 45.

[0131] In some aspects, the disclosure provides a method of treating cancer in a subject, comprising administering to the subject a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0132] (i) a disrupted TRAC gene comprising a nucleic acid sequence at least 90% identical to SEQ ID NO: 44; [0133] (ii) a disrupted β2M gene; and [0134] (iii) a disrupted CD70 gene, [0135] thereby treating the cancer in the subject. In some aspects, the disrupted TRAC gene comprises the nucleic acid sequence set forth in SEQ ID NO: 44.

[0136] In any of the foregoing or related aspects, the engineered T cells are engineered human T cells. In some embodiments, the engineered T cells are engineered allogeneic T cells.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

[0137] FIG. **1** includes a graph showing highly efficient multiple gene editing in TRAC−/β2M−/PD-1−/CD70− (quadruple knockout) T cells.

[0138] FIG. **2** includes a graph showing similar expansion among multigene-edited cells.

[0139] FIG. **3** includes graphs showing efficient multiple gene editing in TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (i.e., 3×KO (CD70), CD70 CAR.sup.+) T cells.

[0140] FIG. **4** includes a graph showing that normal proportions of CD4+ and CD8+ T cells are maintained among the TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cell population.

[0141] FIG. **5** includes a graph showing efficient multiple gene editing in TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells.

[0142] FIG. **6** includes a graph showing that normal proportions of CD4+ and CD8+ T cells are maintained among the TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (i.e., 4×KO, CD70 CAR.sup.+) T cell population.

[0143] FIGS. **7**A-**7**C include graphs showing data relating to the characterization of anti-BCMA CAR+ T cells with multi-gene edits. Double knockout TRAC.sup.−/β2M.sup.−/anti-BCMA CAR.sup.+ T cells and quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-BCMA CAR.sup.+ T cells were stained for TRAC and β2M (FIG. **7**A), PD-1 and CD70 (FIG. **7**B), and BCMA CAR (FIG. **7**C) expression. The anti-BCMA CAR was expressed at approximately 80% in both the double and quadruple knockout CAR T cells.

[0144] FIG. **8** includes flow cytometry plots showing prevention of loss of CD4+ cells in 3× KO (TRAC−/β2M−/CD70−) anti-CD33 CAR T cells compared 2×KO (TRAC−/β2M−) anti-CD33 CAR T cells over three weeks.

[0145] FIG. **9** includes a graph showing CD70 KO enhanced cell proliferation in anti-CD33 CAR T cells over two weeks. The total number of viable cells was quantified in 3×KO (TRAC−/β2M−/CD70−) and 2×KO (TRAC−/β2M−) anti-CD33 CAR T cells.

[0146] FIG. **10** includes a graph showing CD70 KO enhanced cell proliferation in anti-CD19 CAR T cells over two weeks. The total number of viable cells was quantified in 3×KO (TRAC−/β2M−/CD70−) and 2×KO (TRAC−/β2M−) anti-CD33 CAR T cells.

[0147] FIG. **11** includes graphs showing CD70 KO enhanced cell proliferation in anti-BCMA CAR T cells and rescued the detrimental effect of PD1 KO on BCMA CAR cell proliferation. The total number of viable cells was quantified in 4×KO (TRAC−/β2M−/CD70−/PD1−), 3×KO (CD70) (TRAC−/β2M−/CD70−), 3×KO (PD1) (TRAC−/β2M−/PD1−) and 2×KO (TRAC−/β2M−) anti-CD33 CAR T cells.

[0148] FIG. **12** includes graphs showing CD70 KO enhanced cell proliferation in anti-BCMA CAR T cells and rescued the detrimental effect of PD1 KO on BCMA CAR cell proliferation. The total number of viable cells was quantified in 4×KO (TRAC−/β2M−/CD70−/PD1−), 3×KO (CD70) (TRAC−/β2M−/CD70−), 3×KO (PD1) (TRAC−/β2M−/PD1−) and 2×KO (TRAC−/β2M−) anti-CD33 CAR T cells. The anti-BCMA CAR T cells were derived from a different donor T cells as the CAR T cells shown in FIG. **11**.

[0149] FIG. **13** includes a graph showing a comparison of apoptotic cell death due to antigen exposure in 2×KO (TRAC−/B2M−) anti-BCMA CAR+ T cells and 3×KO (TRAC−/B2M−/CD70−) anti-BCMA CAR+ T cells. CAR+ T cells were exposed to plate-bound BCMA antigen for 24 hours with a re-challenge every 24 hours and apoptosis was assessed following each antigen challenge by flow cytometry. Induction of apoptosis due to antigen challenge was lower in anti-BCMA CAR+ T cells with a CD70 KO compared to those without.

[0150] FIG. **14** includes a graph showing a comparison of CAR T cell expansion following antigen

exposure in 2×KO (TRAC−/B2M−) anti-BCMA CAR+ T cells and 3×KO (TRAC−/B2M−/CD70−) anti-BCMA CAR+ T cells. CAR+ T cells were exposed to plate-bound BCMA antigen for 24 hours with a re-challenge every 24 hours and cell expansion was assessed following each antigen challenge and normalized to the population at time 0 h. Population expansion following antigen challenge was higher in anti-BCMA CAR+ T cells with a CD70 KO compared to those without.

[0151] FIG. **15** includes a graph showing robust cell expansion in TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells. The total number of viable cells was quantified in 3×KO (TRAC−/β2M−/CD70−) and 2×KO (TRAC−/β2M−) anti-CD70 CAR T cells. 3×KO cells were generated with either CD70 sgRNA T7 or T8.

[0152] FIG. **16** includes a graph showing robust cell expansion of TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells. The total number of viable cells was quantified in 4×KO (TRAC−/β2M−/PD1−/CD70−), 3×KO (TRAC−/β2M−/PD1−) and 2×KO (TRAC−/β2M−) anti-CD70 CAR T cells.

[0153] FIG. **17** includes graphs showing robust cell killing of both Nalm6 (top panel) cells and Raji (bottom panel) cells by anti-CD19 CAR T cells (TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD19 CAR.sup.+ or TRAC.sup.−/β2M.sup.−/anti-CD19 CAR.sup.+ T cells).

[0154] FIG. **18** includes a graph showing robust cell killing of MV411 cells by anti-CD33 CAR T cells (TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD33 CAR.sup.+ or TRAC.sup.−/β2M.sup.−/anti-CD33 CAR.sup.+ T cells).

[0155] FIG. **19** includes a graph showing robust cell killing of A498 cells by 3×KO (TRAC.sup.−/β2M.sup.−/CD70.sup.−) anti-CD70 CAR.sup.+ T cells compared to 2×KO (TRAC.sup.−/β2M.sup.−) anti-CD70 CAR.sup.+ T cells.

[0156] FIG. **20** includes a graph showing cell expansion of 3×KO (TRAC−/β2M−/CD70−) or 2×KO (TRAC−/β2M−) anti-CD33 CAR T cells after challenge with MV411 target cells.

[0157] FIG. **21** includes a graph showing cell expansion of 3×KO (TRAC−/β2M−/CD70−) or 2×KO (TRAC−/β2M−) anti-CD70 CAR T cells after challenge with Nalm6 target cells.

[0158] FIG. **22**A includes a graph showing A498 cell killing by anti-CD70 CAR T cells after serial rechallenge. 4×KO (TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD1.sup.−), 3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), 3×KO (PD1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−) and 2×KO (TRAC.sup.−/β2M.sup.−) anti-CD70 CAR+ T cells were utilized. 3×KO (CD70), CD70 CAR.sup.+ T cells, and 4×KO, CD70 CAR+ T cells were the most effective. FIG. **22**B includes a graph showing ACHN cell killing by anti-CD70 CAR T cells after serial rechallenge. The same cells as FIG. **22**A were utilized. 3×KO (CD70), CD70 CAR.sup.+ T cells and 4×KO, CD70 CAR.sup.+ T cells were the most effective.

[0159] FIG. **23**A includes a graph showing ACHN cell killing by anti-CD70 CAR T cells at various effector:target ratios. 4×KO (TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD1.sup.−), 3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), 3×KO (PD1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−) and 2×KO (TRAC.sup.−/β2M.sup.−) anti-CD70 CAR+ T cells were utilized. 3×KO (CD70), CD70 CAR.sup.+ T cells and 4×KO, CD70 CAR.sup.+ T cells were superior killers following multiple serial rechallenges. FIG. **23**B includes a graph showing LAG3 (left) and PD1 (right) expression in the cells from FIG. **23**A following eight rechallenges.

[0160] FIGS. **24**A-**24**C include graphs showing that knockout of PD-1 and CD70 enhances cell killing activity of anti-BCMA CAR+ T cells as measured through serial rechallenges with a multiple myeloma cell line (MM.1S). Double knockout (2×KO (TRAC.sup.−/β2M.sup.−) anti-BCMA CAR.sup.+ T cells (circles) began to lose their potency towards MM.1S cells after approximately 4 rechallenges, while quadruple knockout (4×KO (TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD1.sup.−) anti-BCMA CAR.sup.+ T cells (squares) were capable of killing 100% of the MM.1S cells after 10 rechallenges (FIG. **24**A). Consistent with this, the quadruple knockout anti-BCMA CAR.sup.+ T cells continued to secrete IFN-g in response to target cells after 10 rechallenges, while the double knockout anti-BCMA CAR.sup.+ T cells showed reduced IFN-g

secretion after the third rechallenge (FIG. **24**B). The quadruple knockout anti-BCMA CAR.sup.+ T cells also showed higher proliferation in response to exposure to target cells than the double knockout anti-BCMA CAR.sup.+ T cells (FIG. **24**C).

[0161] FIGS. **25**A-**25**C include graphs showing highest cell kill activity in A498 PD-L1 kidney cancer cells (which overexpress PD-L1) using quadruple knockout (4×KO) TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and triple knockout (3×KO (CD70)) TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells, relative to double knockout (2×KO) TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells and triple knockout (3×KO (PD1) TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells. A CAR T cell:A498-PD-L1 cell ratio of 2:1 was used in FIG. **25**A, a CAR T cell:A498-PD-L1 cell ratio of 1:1 was used in FIG. **25**B, and a CAR T cell:A498-PD-L1 cell ratio of 0.5:1 was used in FIG. **25**C.

[0162] FIG. **26**A and FIG. **26**B include graphs showing that quadruple knockout (4×KO) TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells secrete the highest levels of cytokines IFN-gamma (FIG. **26**A) and IL-2 (FIG. **26**B), relative to triple knockout (3×KO (CD70) TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells, double knockout (2×KO) TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells and triple knockout (3×KO (PD1) TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells. A CAR T cell:A498-PD-L1 cell ratio of 1:1 was used.

[0163] FIG. **27**A includes a graph showing results from an experiment designed to assess tumor volume reduction in a subcutaneous A498 renal cell carcinoma model exposed to: 2×KO (TRAC.sup.−/β2M.sup.−), CD70 CAR.sup.+ T cells; 3×KO (PD-1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−), CD70 CAR.sup.+ T cells; 3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), CD70 CAR.sup.+ T cells; or 4×KO (PD-1, CD70) (TRAC.sup.−/β2M.sup.−/PD1.sup.−/CD70.sup.−), CD70 CAR.sup.+ T cells. FIG. **27**B includes a graph showing results from an experiment designed to assess prevention of tumor growth in a subcutaneous A498 renal cell carcinoma rechallenge model. Mice from FIG. **27**A were rechallenged with A498 tumor cells on day 25 and tumor volume was assessed over time. FIG. **27**C includes a graph showing results from an experiment designed to assess tumor volume reduction in a subcutaneous A498 renal cell carcinoma model (large tumor of ˜150 mm3 at time of CAR-T injection) exposed to: 3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), CD70 CAR.sup.+ T cells; 4×KO (PD-1, CD70) (TRAC.sup.−/β2M.sup.−/PD1.sup.−/CD70.sup.−), CD70 CAR.sup.+ T cells; 2×KO (TRAC.sup.−/β2M.sup.−), CD70 CAR.sup.+ T cells; or 3×KO (PD-1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−), CD70 CAR.sup.+ T cells.

[0164] FIG. **28**A includes a graph showing tumor volume reduction in a subcutaneous MM.1S model exposed to: 2×KO (TRAC.sup.−/β2M.sup.−), BCMA CAR.sup.+ T cells; 3×KO (PD-1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−), BCMA CAR.sup.+ T cells; 3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), BCMA CAR.sup.+ T cells; or 4×KO (PD-1, CD70) (TRAC.sup.−/β2M.sup.−/PD1.sup.−/CD70.sup.−), BCMA CAR.sup.+ T cells.

[0165] FIG. **28**B includes a graph showing tumor volume reduction in a subcutaneous MM.1S model following a tumor cell re-challenge. Mice from FIG. **28**A were re-challenged with a second inoculation of MM.1S cells on day 45 and tumor volume was assessed over time.

[0166] FIG. **29** includes graphs showing the number of human CD45.sup.+2×KO (TRAC.sup.−/β2M.sup.−), BCMA CAR.sup.+ T cells; human CD45.sup.+ 3×KO (PD-1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−), BCMA CAR.sup.+ T cells; human CD45.sup.+ 3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), BCMA CAR.sup.+ T cells; and human CD45.sup.+ 4×KO (PD-1, CD70) (TRAC.sup.−/β2M.sup.−/PD1.sup.−/CD70.sup.−), BCMA CAR.sup.+ T cells 1 week (right graph), 2 weeks (middle graph), and 3 weeks (left graph) post dosing.

[0167] FIG. **30** includes graphs showing the results from an experiment designed to assess tumor volume reduction in a subcutaneous RPMI-8226 tumor xenograft model exposed to: TRAC.sup.−/β2M/anti-BCMA CAR.sup.+ T cells (2×KO, BCMA CAR.sup.+ T cells); TRAC.sup.−/β2M.sup.

−/PD1.sup.−/anti-BCMA CAR.sup.+ T cells (3×KO (PD-1), BCMA CAR.sup.+ T cells); TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-BCMA CAR.sup.+ T cells (3×KO (CD70), BCMA CAR.sup.+ T cells); or TRAC.sup.−/β2M.sup.−/PD1.sup.−/CD70.sup.−/anti-BCMA CAR.sup.+ T cells (4×KO (PD-1, CD70), BCMA CAR.sup.+ T cells), at doses of 1×10.sup.5, 3×10.sup.5, 1×10.sup.6, or 3×10.sup.6 cells/mouse.

[0168] FIG. **31** includes a graph showing that TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cell maintain cytokine-dependent proliferation.

[0169] FIG. **32** shows cytokine-dependent growth of the TRAC.sup.−/β2M.sup.−/PD-1.sup. −/CD70.sup.−/anti-CD70 CAR.sup.+ T cells.

[0170] FIG. **33** includes a graph showing 4×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup. −), BCMA CAR.sup.+ T cells maintain cytokine dependency.

[0171] FIG. **34** includes a graph showing enhanced cytokine (IL-2) release by 3×KO (TRAC−/ β2M−/CD70−) anti-CD70 CAR+ T cells compared to 2×KO (TRAC−/β2M−) anti-CD70 CAR+ T cells when co-cultured with A498 kidney cancer cells at various ratios for 24 hours.

[0172] FIG. **35** includes a graph showing robust cell killing of A498 cells by anti-CD70 CAR T cells (2×KO (TRAC.sup.−/β2M.sup.−), CD70 CAR+; 3×KO (PD-1) (TRAC.sup.−/β2M.sup.−/PD-1.sup.−), CD70 CAR+; and 4×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−) CD70 CAR.sup.+) relative to TCR+ T cells. T cells were co-cultured with A498 cells at various ratios for 24 hours and percentage of cell lysis was measured.

[0173] FIG. **36** includes a graph showing highest cell kill activity in A498 kidney cancer cells using quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (4×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (CD70), CD70 CAR+), relative to double knockout TRAC.sup.−/ β2M.sup.−/anti-CD70 CAR.sup.+ (i.e., 2×KO, CD70 CAR.sup.+) T cells and triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ (i.e., 3×KO (PD-1), CD70 CAR.sup.+) T cells. A CAR T cell:A498 cell ratio of 0.25:1 was used. Percentage of cell lysis of A498 cells was measured 24 hours after co-culture.

[0174] FIGS. **37**A and **37**B include graphs showing that quadruple knockout TRAC.sup.−/ β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (4×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (CD70), CD70 CAR+) secrete the highest levels of cytokines IFN-gamma (FIG. **37**A) and IL-2 (FIG. **37**B), relative to double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells (2×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (PD-1), CD70 CAR+). A CAR T cell:A498 cell ratio of 0.25:1 was used. IFN-gamma and IL-2 secretion was measured 24 hours are co-culture.

[0175] FIG. **38** includes a graph showing that knocking out CD70 in anti-CD70 CAR T cells (3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), CD70 CAR+; 3×KO (PD-1) (TRAC.sup.−/ β2M.sup.−/PD1.sup.−), CD70 CAR+; and 4×KO (TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD-1.sup. −), CD70 CAR+) decreased levels of PD-1 expression in CD4+ T cells relative to anti-CD70 CAR T cells expressing endogenous CD70 (2×KO (TRAC.sup.−/β2M.sup.−) CD70 CAR+).

[0176] FIG. **39**A and FIG. **39**B include graphs showing that knocking out CD70 in anti-CD70 CAR T cells (3×KO (CD70) (TRAC.sup.−/β2M.sup.−/CD70.sup.−), CD70 CAR+; 3×KO (PD-1) (TRAC.sup.−/β2M.sup.−/PD1.sup.−), CD70 CAR+; and 4×KO (TRAC.sup.−/β2M.sup. −/CD70.sup.−/PD-1.sup.−), CD70 CAR+) decreased levels of exhaustion marker LAG3 in CD8+ T cells (FIG. **39**A) and CD4+ T cells (FIG. **39**B) relative to anti-CD70 CAR T cells expressing endogenous CD70 (2×KO (TRAC.sup.−/β2M.sup.−) CD70 CAR+).

[0177] FIG. **40**A includes graphs showing relative CD70 expression in five different cancer cell lines (left panel) and relative CD70 expression in three different cancel cell lines (right panel). FIG. **40**B includes graphs showing relative CD70 expression in nine different cancer cell lines. FIGS. **40**C-**40**D include graphs showing highest cell kill activity in ACHN (ATCC® CRL-1611™) kidney

cancer cells (which express low levels of CD70) using quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (4×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (CD70), CD70 CAR+), relative to double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells (2×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (PD-1), CD70 CAR+). A CAR T cell:ACHN cell ratio of 0.5:1 was used in FIG. **40**C and a CAR T cell:ACHN cell ratio of 0.25:1 was used in FIG. **40**D. FIG. **40**E and FIG. **40**F include graphs showing cell kill activity using quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (FIG. **40**E) and triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (FIG. **40**F) against additional solid tumor cell lines with varying levels of CD70 expression (4:1, 1:1, or 0.25:1 effector:target cell ratio). FIG. **40**G includes a graph showing cell kill activity using the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells against solid tumor cell lines after a co-culture period of 24 hours or 96 hours. FIGS. **40**H-**40**J include graphs showing cell kill activity using the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (3KO (CD70), CD70 CAR+) against CD70-deficient chronic myelogenous leukemia (K562) cells (FIG. **40**H), CD70-expressing multiple myeloma (MM.1S) cells (FIG. **40**I), and CD70-expressing T cell lymphoma (HuT78) cells (FIG. **40**J) at various effector:target ratios.

[0178] FIG. **41**A and FIG. **41**B include graphs showing that quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (4×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (CD70), CD70 CAR+) secrete the highest levels of cytokines IFN-gamma (FIG. **41**A) and IL-2 (FIG. **41**B), relative to double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells (2×KO, CD70 CAR+) and triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells (3×KO (PD-1), CD70 CAR+). A CAR T cell:ACHN cell ratio of 0.25:1 was used.

[0179] FIG. **42**A includes a graph showing results from an experiment designed to assess tumor volume reduction in a human ovarian tumor xenograft model (e.g., SKOV-3 tumor cells) exposed to 3×KO (TRAC−/B2M−/CD70−) anti-CD70 CAR T cells. FIG. **42**B includes a graph showing results from an experiment designed to assess tumor volume reduction in a human non-small cell lung tumor xenograft model (e.g., NCI-H1975 tumor cells) exposed to 3×KO (TRAC−/B2M−/CD70−) anti-CD70 CAR T cells. FIG. **42**C includes a graph showing results from an experiment designed to assess tumor volume reduction in a human pancreatic tumor xenograft model (e.g., Hs766T tumor cells) exposed to 3×KO (TRAC−/B2M−/CD70−) anti-CD70 CAR T cells. FIG. **42**D includes graphs showing results from an experiment designed to assess tumor volume reduction in a human T-cell lymphoma xenograft model (e.g., HuT78 tumor cells) exposed to 3×KO (TRAC−/B2M−/CD70−) anti-CD70 CAR T cells. Tumor volumes of individual mice (left) and mean tumor volumes (right) are shown.

DETAILED DESCRIPTION

[0180] The present disclosure is based, at least in part, on the discovery that disrupting the CD70 gene in immune cells engineered to express an antigen targeting moiety (e.g., a CAR) enhances several characteristics important for cell-based immunotherapy, including anti-tumor efficacy. Specifically, such engineered immune cells showed unexpected superior features, including extended proliferation and in vivo persistence resulting in long-term, enhanced anti-tumor efficacy. Notably, these unexpected features have been demonstrated with targeting moieties specific for various antigens, including BCMA, CD19, CD33 and CD70.

[0181] As demonstrated herein, disrupting the CD70 gene resulted in maintenance of cytotoxicity of immune cells engineered to express an antigen targeting moiety after multiple rounds of challenges by cancer cells in vitro. Without wishing to be bound by theory, this maintenance of cytotoxicity indicates disrupting the CD70 gene makes the engineered immune cells resistant to exhaustion and may result in cells that live longer.

[0182] It was also found that disrupting the CD70 gene in immune cells engineered to express an antigen targeting moiety enhanced anti-tumor efficacy against large tumors and induced a durable anti-cancer memory response. Specifically, the anti-cancer memory response prevented tumor growth upon re-challenge. Further, it has been demonstrated disrupting the CD70 gene results in enhanced cytotoxicity of immune cells engineered to express an antigen targeting moiety at lower ratios of engineered immune cells to target cells, indicating the potential efficacy of low doses of engineered immune cells.

[0183] It has also been shown disruption of the CD70 gene enhances cell proliferation and in vivo persistence of engineered immune cells. Without wishing to be bound by theory, it is believed the superior features of the engineered immune cells described herein allow for more consistent cell populations, larger scale production due to the cells' ability to survive more cell division, and fewer starting cells required to produce the engineered cells. Such features may also prove beneficial in a clinical setting. For example, increased expansion and decreased exhaustion indicates increased efficacy per dose and the ability to obtain efficacy with lower doses.

[0184] It has also been demonstrated that disrupting the CD70 gene in immune cells engineered to express an antigen targeting moiety maintains cytotoxicity against cancer cells expressing highly immune suppressive molecules, i.e., PD-L1. Without wishing to be bound by theory, it is believed the internal negative signal of PD-1 expressed on immune cells when bound to PD-L1 expressed on cancer cells, is overcome by disrupting CD70.

[0185] Accordingly, provided herein are methods and compositions (e.g., cell compositions) for the treatment of cancer, such as BCMA.sup.+, CD19.sup.+, CD33.sup.+, and CD70.sup.+ malignancies, involving the use of the engineered immune cells with increased efficacy and persistence.

CD70 Gene Edit

[0186] Cluster of Differentiation 70 (CD70) is a member of the tumor necrosis factor superfamily and its expression is restricted to activated T and B lymphocytes and mature dendritic cells. CD70 is implicated in tumor cell and regulatory T cell survival through interaction with its ligand, CD27. CD70 and its receptor CD27 have multiple roles in immune function in multiple cell types including T cells (activated and T regs), and B cells. It is unclear exactly how CD70 functions in all of these cell types to control functions such as apoptosis, with publications indicating contradicting roles. For example, it has been reported that CD70 induces apoptosis or survival of T cells depending on the antigenic load (Wensveen, F., et al. J. Immunol, Vol 188: 4256-4267, 2012).

[0187] While CAR T cells have proved to be an effective immunotherapeutic, various challenges remain. For example, over time CAR T cells become exhausted and become ineffective in vivo. With regards to manufacturing, it takes significant time to produce enough cells to dose a patient. To address these limitations, the present disclosure provides CAR T cells that have been engineered to disrupt endogenous CD70 expression while at the same time expressing an antigen targeting moiety (e.g., an scFv).

[0188] Surprisingly, the present disclosure shows disrupting the CD70 gene enables increased CAR T health and function (e.g., extended proliferation, reduced exhaustion) regardless of the antigen being targeted by the scFv in the CAR T. This applies even to antigens expressed on T cells such as CD33 and CD70 where the effects of the disrupted CD70 gene retain CAR T function even where fratricide may be expected. That is, these CD70 knockout cells (e.g., in which the CD70 gene has been edited using CRISPR/Cas9 gene editing technology), independent of the CAR insertion, exhibit continued, steady cell growth, relative to unmodified T cells (or edited T cells that express CD70) and express lower levels of exhaustion markers, such as LAG3. The CAR T cells of the present disclosure, may include any antibody (including whole antibodies and antibody fragments) or other molecule (e.g., receptor or ligand) that specifically binds to a cancer antigen to guide the CAR T cell to a cancer cell. In some embodiments, the antibody is an anti-CD70 antibody (e.g., an anti-CD70 scFv). In other embodiments, the antibody is an anti-CD19 antibody (e.g., an anti-CD19

scFv). In yet other embodiments, the antibody is an anti-BCMA antibody (e.g., an anti-BCMA scFv). In other embodiments, the antibody is an anti-CD33 antibody (e.g., an anti-CD33 scFv). Other cancer antigens are encompassed by the present disclosure.

[0189] It should be understood that gene disruption encompasses gene modification through gene editing (e.g., using CRISPR/Cas gene editing to insert or delete one or more nucleotides). In some embodiments, a disrupted gene is a gene that does not encode functional protein. In some embodiments, a cell that comprises a disrupted gene does not express (e.g., at the cell surface) a detectable level (e.g. by antibody, e.g., by flow cytometry) of the protein encoded by the gene. A cell that does not express a detectable level of the protein may be referred to as a knockout cell. For example, a cell having a CD70 gene edit may be considered a CD70 knockout cell if CD70 protein cannot be detected at the cell surface using an antibody that specifically binds CD70 protein.

[0190] Provided herein, in some embodiments, are populations of cells in which a certain percentage of the cells has been edited (e.g., CD70 gene edited), resulting in a certain percentage of cells not expressing a particular gene and/or protein. In some embodiments, at least 50% (e.g., 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 85%) of the cells of a gene-edited population of cells are CD70 knockout cells. In some embodiments, at least 50% of the cells (e.g. T cells) of the population do not express detectable levels of CD70 protein. In some embodiments, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the cells of a gene-edited population of cells may be CD70 knockout cells.

[0191] In some embodiments, 10%, 15%, 20%, 25%, 30%, 35% or 40% of the engineered T cells of a population do not express a detectable level of CD70 surface protein. In some embodiments, the percent of engineered T cells that do not express a detectable level of CD70 surface protein increases over time. Thus, in some embodiments, at least 50% of the engineered T cells of a population of engineered T cells does not express a detectable level of CD70 surface protein. For example, at least 55%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the engineered T cells of a population may not express a detectable level of CD70 surface protein. In some embodiments, 50%-100%, 50%-90%, 50%-80%, 50%-70%, 50%-60%, 60%-100%, 60%-90%, 60%-80%, 60%-70%, 70%-100%, 70%-90%, 70%-80%, 80%-100%, 80%-90%, or 90%-100% of the engineered T cells of a population does not express a detectable level of CD70 surface protein.

[0192] Non-limiting examples of modified and unmodified CD70 gRNA sequences that may be used as provided herein to create a genomic alteration (e.g., disruption, e.g., deletion, insertion, substitution) in the CD70 gene are listed in Table 5 (e.g., SEQ ID NOS: 23-29 and 33-39). Other gRNA sequences may be designed using the CD70 gene sequence located on Chromosome 19 (GRCh38 coordinates: Chromosome 19: 6,583,183-6,604,103; Ensembl: ENSG00000125726). In certain embodiments, gRNAs targeting the CD70 genomic region create Indels (e.g.: insertions, deletions or substitutions) in, or around, the CD70 gene disrupting expression of the CD70 mRNA and/or protein.

[0193] In some embodiments, a ribonucleoprotein particle (RNP) containing an RNA-guided nuclease (e.g., a Cas nuclease, such as a Cas9 nuclease) and a gRNA targeting the CD70 gene (or any other gene of interest) are delivered to T cells (e.g., primary T cells). In other embodiments, the RNA-guided nuclease and gRNA are delivered separately to T cells. A ribonucleoprotein particle (RNP) is simply an RNA-guided nuclease (e.g., Cas9) pre-complexed/complexed with (bound to) a gRNA.

[0194] In some embodiments, the gRNA targeting the CD70 gene is a synthetic modified gRNA such as but not limited to any one of the gRNAs comprising SEQ ID NO: 33-39. In some embodiments, the gRNA targeting the CD70 gene is a synthetic unmodified gRNA such as but not limited to any one of the gRNAs comprising SEQ ID NO: 23-29.

[0195] In some embodiments, gRNAs targeting the CD70 genomic region and RNA-guided nuclease create double stranded breaks in the CD70 gene. Repair of the break results in Indels in

the CD70 gene wherein the CD70 gene sequence may comprises a nucleotide sequence selected from the group consisting of: SEQ ID NOs: 129-134.

Multi-Gene Editing

[0196] The engineered T cells of the present disclosure, in some embodiments, include more than one disrupted gene (e.g.: more than one gene edit), for example, in more than one gene. For example, an engineered T cell may comprise a disrupted CD70 gene, a disrupted T cell receptor alpha chain constant region (TRAC) gene, a disrupted beta-2-microglobulin (β2M) gene, a disrupted programmed cell death-1 (PD-1 or PDCD1) gene, or any combination of two or more of the foregoing disrupted genes. In some embodiments, an engineered T cell comprises a disrupted TRAC gene, a disrupted β2M gene, and a disrupted CD70 gene. In some embodiments, an engineered T cell comprises a disrupted TRAC gene, a disrupted β2M gene, and a disrupted PD-1 gene. In some embodiments, an engineered T cell comprises a disrupted TRAC gene, a disrupted β2M gene, a disrupted CD70 gene and a disrupted PD-1 gene.

TRAC Gene Edit

[0197] In some embodiments, an engineered T cell comprises a disrupted TRAC gene. This disruption leads to loss of function of the TCR and renders the engineered T cell non-alloreactive and suitable for allogeneic transplantation, minimizing the risk of graft versus host disease. In some embodiments, expression of the endogenous TRAC gene is eliminated to prevent a graft-versus-host response.

[0198] In some embodiments, a disruption in the TRAC gene expression is created by knocking a chimeric antigen receptor (CAR) into the TRAC gene (e.g., using an adeno-associated viral (AAV) vector and donor template). In some embodiments, a disruption in the TRAC gene expression is created with a nuclease and gRNAs targeting the TRAC genomic region. In some embodiments, a genomic deletion in the TRAC gene is created by HDR, wherein a chimeric antigen receptor (CAR) replaces a segment of the TRAC gene (e.g., using an adeno-associated viral (AAV) vector and donor template). In some embodiments, a disruption in the TRAC gene expression is created with a nuclease and gRNAs targeting the TRAC genomic region, and knocking a chimeric antigen receptor (CAR) into the TRAC gene.

[0199] Non-limiting examples of modified and unmodified TRAC gRNA sequences that may be used as provided herein to create a genomic in the TRAC gene are listed in Table 7 (e.g., SEQ ID NOS: 30 and 40). See also International Application No. PCT/US2018/032334, filed May 11, 2018, incorporated herein by reference. Other gRNA sequences may be designed using the TRAC gene sequence located on chromosome 14 (GRCh38: chromosome 14: 22,547,506-22,552,154; Ensembl; ENSG00000277734). In some embodiments, gRNAs targeting the TRAC genomic region and RNA-guided nuclease create breaks in the TRAC genomic region resulting Indels in the TRAC gene disrupting expression of the mRNA or protein.

[0200] In some embodiments, at least 50% of the engineered T cells of a population do not express a detectable level of T cell receptor (TCR) surface protein. For example, at least 55%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the engineered T cells of a population may not express a detectable level of TCR surface protein. In some embodiments, 50%-100%, 50%-90%, 50%-80%, 50%-70%, 50%-60%, 60%-100%, 60%-90%, 60%-80%, 60%-70%, 70%-100%, 70%-90%, 70%-80%, 80%-100%, 80%-90%, or 90%-100% of the engineered T cells of a population do not express a detectable level of TCR surface protein.

[0201] In some embodiments, a ribonucleoprotein particle (RNP) containing an RNA-guided nuclease (e.g., a Cas nuclease, such as a Cas9 nuclease) and a gRNA targeting the TRAC gene (or any other gene of interest) are delivered to T cells (e.g., primary T cells). In other embodiments, the RNA-guided nuclease and gRNA are delivered separately to T cells. A ribonucleoprotein particle (RNP) is simply an RNA-guided nuclease (e.g., Cas9) pre-complexed/complexed with a gRNA.

[0202] In some embodiments, gRNAs and RNA-guided nuclease targeting the TRAC genomic

region result Indels in the TRAC gene comprising a nucleotide sequence selected from the following sequences in Table 1:

TABLE-US-00001 TABLE 1 SEQ ID Sequence NO: AAGAGCAACAAATCTGACT 1 AAGAGCAACAGTGCTGTGCCTGGAGCAACAAATCTGACT 2 AAGAGCAACAAATCTGACT AAGAGCAACAGTGCTGGAGCAACAAATCTGACT 3 AAGAGCAACAAATCTGACT AAGAGCAACAGTGCCTGGAGCAACAAATCTGACT 4 AAGAGCAACAAATCTGACT AAGAGCAACAGTGCTGACTAAGAGCAACAAATCTGACT 5 AAGAGCAACAGTGCTGTGGGCCTGGAGCAACAAATCTGACT 6 AAGAGCAACAAATCTGACT AAGAGCAACAGTGCTGGCCTGGAGCAACAAATCTGACT 7 AAGAGCAACAAATCTGACT AAGAGCAACAGTGCTGTGTGCCTGGAGCAACAAATCTGACT 8 AAGAGCAACAAATCTGACT

[0203] In some embodiments, an engineered T cell comprises a deletion in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion of 15-30 base pairs in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 base pairs in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion of more than 30 base pairs in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion of 20 base pairs in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion of SEQ ID NO: 86 in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion comprising SEQ ID NO: 86 in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion of SEQ ID NO: 118 in the TRAC gene relative to unmodified T cells. In some embodiments, an engineered T cell comprises a deletion comprising SEQ ID NO: 118 in the TRAC gene relative to unmodified T cells.

β2M Gene Edit

[0204] In some embodiments, an engineered T cell comprises a disrupted β2M gene. β2M is a common (invariant) component of MHC I complexes. Disrupting its expression by gene editing will prevent host versus therapeutic allogeneic T cells responses leading to increased allogeneic T cell persistence. In some embodiments, expression of the endogenous β2M gene is eliminated to prevent a host-versus-graft response.

[0205] Non-limiting examples of modified and unmodified β2M gRNA sequences that may be used as provided herein to create a genomic deletion in the β2M gene are listed in Table 7 (e.g., SEQ ID NOS: 31 and 41). See also International Application No. PCT/US2018/032334, filed May 11, 2018, incorporated herein by reference. Other gRNA sequences may be designed using the β2M gene sequence located on Chromosome 15 (GRCh38 coordinates: Chromosome 15: 44,711,477-44,718,877; Ensembl: ENSG00000166710).

[0206] In some embodiments, gRNAs targeting the β2M genomic region and RNA-guided nuclease create breaks in the β2M genomic region resulting in Indels in the β2M gene disrupting expression of the mRNA or protein.

[0207] In some embodiments, at least 50% of the engineered T cells of a population do not express a detectable level of β2M surface protein. For example, at least 55%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the engineered T cells of a population may not express a detectable level of β2M surface protein. In some embodiments, 50%-100%, 50%-90%, 50%-80%, 50%-70%, 50%-60%, 60%-100%, 60%-90%, 60%-80%, 60%-70%, 70%-100%, 70%-90%, 70%-80%, 80%-100%, 80%-90%, or 90%-100% of the engineered T cells of a population do not express a detectable level of β2M surface protein.

[0208] In some embodiments, less than 50% of the engineered T cells of a population of cells express a detectable level of β2M surface protein. In some embodiments, less than 30% of the

engineered T cells of a population of cells express a detectable level of β2M surface protein. For example, less than 50%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, or less than 5% of the engineered T cells of a population of cells express a detectable level of β2M surface protein. In some embodiments, 40%-30%, 40%-20%, 40%-10%, 40%-5%, 30%-20%, 30%-10%, 30%-5%, 20%-10%, 20%-5%, or 10%-5% of the engineered T cells of a population of cells express a detectable level of β2M surface protein.

[0209] In some embodiments, a ribonucleoprotein particle (RNP) containing an RNA-guided nuclease (e.g., a Cas nuclease, such as a Cas9 nuclease) and a gRNA targeting the B2M gene (or any other gene of interest) are delivered to T cells (e.g., primary T cells). In other embodiments, the RNA-guided nuclease and gRNA are delivered separately to T cells. A ribonucleoprotein particle (RNP) is simply a RNA-guided nuclease (e.g., Cas9) pre-complexed/complexed with a gRNA.

[0210] In some embodiments, an edited β2M gene comprises a nucleotide sequence selected from the following sequences in Table 2.

TABLE-US-00002 TABLE 2

SEQ ID	Sequences	NO:
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGCCTGGA GGCTATCCAGCGTGAGTCTCTCCTACCCTCCCGCT	9
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCGCCTGGAG GCTATCCAGCGTGAGTCTCTCCTACCCTCCCGCT	10
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGAGGCT ATCCAGCGTGAGTCTCTCCTACCCTCCCGCT	11
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGATAGC CTGGAGGCTATCCAGCGTGAGTCTCTCCTACCCTCCCGCT	12
CGTGGCCTTAGCTGTGCTCGCGCTATCCAGCGTGAGTCTCTCCT ACCCTCCCGCT	13
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGTGGCCT GGAGGCTATCCAGCGTGAGTCTCTCCTACCCTCCCGCT	14

PD-1 Gene Edit

[0211] PD-1 is an immune checkpoint molecule that is upregulated in activated T cells and serves to dampen or stop T cell responses. Disrupting PD-1 by gene editing could lead to more persistent and/or potent therapeutic T cell responses and/or reduce immune suppression in a subject. In some embodiments, an engineered T cell comprises a disrupted PD-1 gene. In some embodiments, expression of the endogenous PD-1 gene is eliminated to enhance anti-tumor efficacy of the CAR T cells of the present disclosure.

[0212] Non-limiting examples of modified and unmodified PD-1 gRNA sequences that may be used as provided herein to create a genomic deletion in the PD-1 gene are listed in Table 5 (e.g., SEQ ID NOS: 32 and 42). See also International Application No. PCT/US2018/032334, filed May 11, 2018, incorporated herein by reference. Other gRNA sequences may be designed using the PD-1 gene sequence located on Chromosome 2 (GRCh38 coordinates: Chromosome 2: 241,849,881-241,858,908; Ensembl: ENSG00000188389).

[0213] In some embodiments, gRNAs targeting and RNA-guided nuclease the PD-1 genomic region create breaks in the TRAC genomic region resulting in Indels in the PD-1 gene disrupting expression of the PD-1 mRNA or protein.

[0214] In some embodiments, at least 50% of the engineered T cells of a population do not express a detectable level of PD-1 surface protein. For example, at least 55%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the engineered T cells of a population may not express a detectable level of PD-1 surface protein. In some embodiments, 50%-100%, 50%-90%, 50%-80%, 50%-70%, 50%-60%, 60%-100%, 60%-90%, 60%-80%, 60%-70%, 70%-100%, 70%-90%, 70%-80%, 80%-100%, 80%-90%, or 90%-100% of the engineered T cells of a population do not express a detectable level of PD-1 surface protein.

[0215] In some embodiments, a ribonucleoprotein particle (RNP) containing an RNA-guided nuclease (e.g., a Cas nuclease, such as a Cas9 nuclease) and a gRNA targeting the PD-1 gene (or

any other gene of interest) are delivered to T cells (e.g., primary T cells). In other embodiments, the RNA-guided nuclease and gRNA are delivered separately to T cells. A ribonucleoprotein particle (RNP) is simply an RNA-guided nuclease (e.g., Cas9) pre-complexed/complexed with a gRNA.

Cellular Phenotypes

[0216] In some embodiments, one or more gene edits within a population of cells results in a phenotype associated with changes in cellular proliferative capacity, cellular exhaustion, cellular viability, cellular lysis capability (e.g., increase cytokine production and/or release), or any combination thereof.

[0217] In some embodiments, engineered T cells of a population comprise a CAR that includes an anti-CD70 scFv ectodomain. In some embodiments, engineered T cells of a population comprise a CAR that includes an anti-BCMA scFv ectodomain. In some embodiments, engineered T cells of a population comprise a CAR that includes an anti-CD19 scFv ectodomain. In some embodiments, engineered T cells of a population comprise a CAR that includes an anti-CD33 scFv ectodomain. Any of the foregoing engineered T cells may also comprise a disruption in one or more of the following genes: TRAC, β2M, PD-1, and/or CD70 (e.g., TRAC.sup.−/β2M.sup.−/CD70.sup.−; TRAC.sup.−/β2M.sup.−/PD-1.sup.−; or TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−).

[0218] In some embodiments, engineered T cells of the present disclosure exhibit increased cellular proliferative capacity relative to control cells. In some embodiments, engineered T cells of the present disclosure exhibit at least 20% greater cellular proliferative capacity, relative to control T cells. For example, engineered T cells (e.g., TRAC.sup.−/β2M.sup.−/CD70.sup.−; TRAC.sup.−/β2M.sup.−/PD-1.sup.−; or TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−; with or without a CAR) may exhibit at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, or at least 90% greater cellular proliferative capacity, relative to control T cells. In some embodiments, engineered T cells of the present disclosure exhibit 20%-100%, 20%-90%, 20%-80%, 20%-70%, 20%-60%, 20%-50%, 30%-100%, 30%-90%, 30%-80%, 30%-70%, 30%-60%, 30%-50%, 40%-100%, 40%-90%, 40%-80%, 40%-70%, 40%-60%, 40%-50%, 50%-100%, 50%-90%, 50%-80%, 50%-70%, or 50%-60% greater cellular proliferative capacity, relative to control T cells. Methods of measuring cell proliferation are known to those of skill in the art and described herein.

[0219] In some embodiments, engineered T cells of the present disclosure exhibit reduced exhaustion, relative to control T cells. For example, the engineered T cells may express reduced levels of LAG3 (or other exhaustion markers), relative to control T cells. In some embodiments, the levels of LAG3 expression are reduced by at least 20%, relative to control T cells. For example, the levels of LAG3 expression may be reduced by at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, or at least 90%, relative to control T cells. In some embodiments, the levels of LAG3 expression are reduced by 20%-100%, 20%-90%, 20%-80%, 20%-70%, 20%-60%, 20%-50%, 30%-100%, 30%-90%, 30%-80%, 30%-70%, 30%-60%, 30%-50%, 40%-100%, 40%-90%, 40%-80%, 40%-70%, 40%-60%, 40%-50%, 50%-100%, 50%-90%, 50%-80%, 50%-70%, or 50%-60%, relative to control T cells. In some embodiments, reduced exhaustion is determined by measuring decreased surface expression of exhaustion markers, including TIGIT, PD-1, LAG-3 or combinations thereof. Methods for measuring surface expression are known to those of skill in the art and described herein.

[0220] In some embodiments, engineered T cells of the present disclosure exhibit increased cellular viability relative to control cells. In some embodiments, engineered T cells of the present disclosure exhibit an at least 20% increase in cellular viability, relative to control cells. For example, engineered T cells of the present disclosure may exhibit at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, or at least 90% increase in cellular viability, relative to control cells. In some embodiments, engineered T cells of the present disclosure exhibit a 20%-100%, 20%-90%,

20%-80%, 20%-70%, 20%-60%, 20%-50%, 30%-100%, 30%-90%, 30%-80%, 30%-70%, 30%-60%, 30%-50%, 40%-100%, 40%-90%, 40%-80%, 40%-70%, 40%-60%, 40%-50%, 50%-100%, 50%-90%, 50%-80%, 50%-70%, or 50%-60% increase in cellular viability, relative to control cells. Methods of measuring cell viability are known to those of skill in the art and described herein.

[0221] In some embodiments, engineered T cells of the present disclosure exhibit increased cellular lysis capability relative to control cells. In some embodiments, engineered T cells of the present disclosure exhibit an at least 20% increase in cellular lysis capability (kill at least 20% more target cells), relative to control cells. For example, engineered T cells of the present disclosure may exhibit an at least at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, or at least 90% increase in cellular lysis capability, relative to control cells. In some embodiments, engineered T cells of the present disclosure exhibit a 20%-100%, 20%-90%, 20%-80%, 20%-70%, 20%-60%, 20%-50%, 30%-100%, 30%-90%, 30%-80%, 30%-70%, 30%-60%, 30%-50%, 40%-100%, 40%-90%, 40%-80%, 40%-70%, 40%-60%, 40%-50%, 50%-100%, 50%-90%, 50%-80%, 50%-70%, or 50%-60% increase in cellular lysis capability, relative to control cells.

[0222] In some embodiments, engineered T cells of the present disclosure exhibit increased cytokine secretion relative to control cells. For example, in some embodiments the level of cytokines (e.g., IL-2 and/or IFN-gamma) secreted by the engineered T cells is at least 2-fold (e.g., at least 3-fold, at least 4-fold, or at least 5-fold) greater than the level of cytokines secreted by control T cells.

[0223] Control T cells, in some embodiments, are engineered T cells (e.g., gene edited T cells) that express endogenous CD70 protein (CD70 normally expressed by T cells). In some embodiments, control T cells are engineered T cells that express endogenous CD70 protein and comprise a TRAC gene disrupted by insertion of a nucleic acid encoding a CAR (e.g., an anti-CD70 CAR or anti-BCMA CAR), a disrupted β2M gene, a disrupted PD-1 gene, or any combination of the foregoing disrupted genes. In some embodiments, control T cells are unedited T cells.

[0224] Surprisingly, the multi-gene edited CAR T cells of the present disclosure (e.g., TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.− cells) maintain cytotoxicity (ability to kill cancer cells), following multiple challenges (also referred to as rechallenges(s)) with cancer cells. In some embodiments, the engineered T cells maintain cytotoxicity following at least 1 rechallenge with a target cell, wherein the target cell expresses an antigen recognized by the CAR T cells. In some embodiments, the engineered T cells maintain cytotoxicity following at least 2 rechallenges with a target cell, wherein the target cell expresses an antigen recognized by the CAR T cells. In some embodiments, the engineered T cells maintain cytotoxicity following at least 1 rechallenge with a cancer cell. In some embodiments, the engineered T cells maintain cytotoxicity following at least 2 rechallenges with a cancer cell. In some embodiments, the engineered T cells maintain cytotoxicity following 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 rechallenges with a target cell, wherein the target cell expresses an antigen recognized by the CAR T cells. In some embodiments, the engineered T cells maintain cytotoxicity following 2, 3, 4, 5, 6, 7, 8, 9, or 10 rechallenges with a target cell, wherein the target cell expresses an antigen recognized by the CAR T cells. In some embodiments, the engineered T cells maintain cytotoxicity following 2, 3, 4, 5, 6, 7, 8, 9, or 10 rechallenges with a cancer cell. In some embodiments, the engineered T cells maintain cytotoxicity following 10 or more rechallenges with a a target cell, wherein the target cell expresses an antigen recognized by the CAR T cells. In some embodiments, the engineered T cells maintain cytotoxicity following 10 or more rechallenges with a cancer cell. In some embodiments, the engineered T cells express a CAR specific for CD70 and the target cell (e.g., cancer cell) expresses CD70. In some embodiments, the engineered T cells express a CAR specific for CD19 and the target cell (e.g., cancer cell) expresses CD19. In some embodiments, the engineered T cells express a CAR specific for CD33 and the target cell (e.g., cancer cell) expresses CD33. In some embodiments, the

engineered T cells express a CAR specific for BCMA and the target cell (e.g., cancer cell) expresses BCMA.

Gene Editing Methods

[0225] Gene editing (including genomic editing) is a type of genetic engineering in which nucleotide(s)/nucleic acid(s) is/are inserted, deleted, and/or substituted in a DNA sequence, such as in the genome of a targeted cell. Targeted gene editing enables insertion, deletion, and/or substitution at pre-selected sites in the genome of a targeted cell (e.g., in a targeted gene or targeted DNA sequence). When a sequence of an endogenous gene is edited, for example by deletion, insertion or substitution of nucleotide(s)/nucleic acid(s), the endogenous gene comprising the affected sequence may be knocked-out or knocked-down due to the sequence alteration. Therefore, targeted editing may be used to disrupt endogenous gene expression. "Targeted integration" refers to a process involving insertion of one or more exogenous sequences, with or without deletion of an endogenous sequence at the insertion site. Targeted integration can result from targeted gene editing when a donor template containing an exogenous sequence is present. As used herein, a "disrupted gene" refers to a gene comprising an insertion, deletion or substitution relative to an endogenous gene such that expression of a functional protein from the endogenous gene is reduced or inhibited. As used herein, "disrupting a gene" refers to a method of inserting, deleting or substituting at least one nucleotide/nucleic acid in an endogenous gene such that expression of a functional protein from the endogenous gene is reduced or inhibited. Methods of disrupting a gene are known to those of skill in the art and described herein.

[0226] Targeted editing can be achieved either through a nuclease-independent approach, or through a nuclease-dependent approach. In the nuclease-independent targeted editing approach, homologous recombination is guided by homologous sequences flanking an exogenous polynucleotide to be introduced into an endogenous sequence through the enzymatic machinery of the host cell. The exogenous polynucleotide may introduce deletions, insertions or replacement of nucleotides in the endogenous sequence.

[0227] Alternatively, the nuclease-dependent approach can achieve targeted editing with higher frequency through the specific introduction of double strand breaks (DSBs) by specific rare-cutting nucleases (e.g., endonucleases). Such nuclease-dependent targeted editing also utilizes DNA repair mechanisms, for example, non-homologous end joining (NHEJ), which occurs in response to DSBs. DNA repair by NHEJ often leads to random insertions or deletions (indels) of a small number of endogenous nucleotides. In contrast to NHEJ mediated repair, repair can also occur by a homology directed repair (HDR). When a donor template containing exogenous genetic material flanked by a pair of homology arms is present, the exogenous genetic material can be introduced into the genome by HDR, which results in targeted integration of the exogenous genetic material.

[0228] Available endonucleases capable of introducing specific and targeted DSBs include, but not limited to, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and RNA-guided CRISPR-Cas9 nuclease (CRISPR/Cas9; Clustered Regular Interspaced Short Palindromic Repeats Associated 9). Additionally, DICE (dual integrase cassette exchange) system utilizing phiC31 and Bxb1 integrases may also be used for targeted integration.

[0229] ZFNs are targeted nucleases comprising a nuclease fused to a zinc finger DNA binding domain (ZFBD), which is a polypeptide domain that binds DNA in a sequence-specific manner through one or more zinc fingers. A zinc finger is a domain of about 30 amino acids within the zinc finger binding domain whose structure is stabilized through coordination of a zinc ion. Examples of zinc fingers include, but not limited to, C2H2 zinc fingers, C3H zinc fingers, and C4 zinc fingers. A designed zinc finger domain is a domain not occurring in nature whose design/composition results principally from rational criteria, e.g., application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP designs and binding data. See, for example, U.S. Pat. Nos. 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496. A selected zinc

finger domain is a domain not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. ZFNs are described in greater detail in U.S. Pat. Nos. 7,888,121 and 7,972,854. The most recognized example of a ZFN is a fusion of the FokI nuclease with a zinc finger DNA binding domain.

[0230] A TALEN is a targeted nuclease comprising a nuclease fused to a TAL effector DNA binding domain. A "transcription activator-like effector DNA binding domain", "TAL effector DNA binding domain", or "TALE DNA binding domain" is a polypeptide domain of TAL effector proteins that is responsible for binding of the TAL effector protein to DNA. TAL effector proteins are secreted by plant pathogens of the genus *Xanthomonas* during infection. These proteins enter the nucleus of the plant cell, bind effector-specific DNA sequences via their DNA binding domain, and activate gene transcription at these sequences via their transactivation domains. TAL effector DNA binding domain specificity depends on an effector-variable number of imperfect 34 amino acid repeats, which comprise polymorphisms at select repeat positions called repeat variable-diresidues (RVD). TALENs are described in greater detail in US Patent Application No. 2011/0145940. The most recognized example of a TALEN in the art is a fusion polypeptide of the FokI nuclease to a TAL effector DNA binding domain.

[0231] Additional examples of targeted nucleases suitable for use as provided herein include, but are not limited to, Bxb1, phiC31, R4, PhiBT1, and WP/SPBc/TP901-1, whether used individually or in combination.

[0232] Other non-limiting examples of targeted nucleases include naturally-occurring and recombinant nucleases, e.g., CRISPR/Cas9, restriction endonucleases, meganucleases homing endonucleases, and the like.

CRISPR-Cas9 Gene Editing

[0233] The CRISPR-Cas9 system is a naturally-occurring defense mechanism in prokaryotes that has been repurposed as an RNA-guided DNA-targeting platform used for gene editing. It relies on the DNA nuclease Cas9, and two noncoding RNAs, crisprRNA (crRNA) and trans-activating RNA (tracrRNA), to target the cleavage of DNA. CRISPR is an abbreviation for Clustered Regularly Interspaced Short Palindromic Repeats, a family of DNA sequences found in the genomes of bacteria and archaea that contain fragments of DNA (spacer DNA) with similarity to foreign DNA previously exposed to the cell, for example, by viruses that have infected or attacked the prokaryote. These fragments of DNA are used by the prokaryote to detect and destroy similar foreign DNA upon re-introduction, for example, from similar viruses during subsequent attacks. Transcription of the CRISPR locus results in the formation of an RNA molecule comprising the spacer sequence, which associates with and targets Cas (CRISPR-associated) proteins able to recognize and cut the foreign, exogenous DNA. Numerous types and classes of CRISPR/Cas systems have been described (see e.g., Koonin et al., (2017) Curr Opin Microbiol 37:67-78).

[0234] crRNA drives sequence recognition and specificity of the CRISPR-Cas9 complex through Watson-Crick base pairing typically with a 20 nucleotide (nt) sequence in the target DNA. Changing the sequence of the 5′ 20 nt in the crRNA allows targeting of the CRISPR-Cas9 complex to specific loci. The CRISPR-Cas9 complex only binds DNA sequences that contain a sequence match to the first 20 nt of the crRNA, single-guide RNA (sgRNA), if the target sequence is followed by a specific short DNA motif (with the sequence NGG) referred to as a protospacer adjacent motif (PAM).

[0235] TracrRNA hybridizes with the 3′ end of crRNA to form an RNA-duplex structure that is bound by the Cas9 endonuclease to form the catalytically active CRISPR-Cas9 complex, which can then cleave the target DNA.

[0236] Once the CRISPR-Cas9 complex is bound to DNA at a target site, two independent nuclease domains within the Cas9 enzyme each cleave one of the DNA strands upstream of the PAM site, leaving a double-strand break (DSB) where both strands of the DNA terminate in a base pair (a blunt end).

[0237] After binding of CRISPR-Cas9 complex to DNA at a specific target site and formation of the site-specific DSB, the next key step is repair of the DSB. Cells use two main DNA repair pathways to repair the DSB: non-homologous end-joining (NHEJ) and homology-directed repair (HDR).

[0238] NHEJ is a robust repair mechanism that appears highly active in the majority of cell types, including non-dividing cells. NHEJ is error-prone and can often result in the removal or addition of between one and several hundred nucleotides at the site of the DSB, though such modifications are typically <20 nt. The resulting insertions and deletions (indels) can disrupt coding or noncoding regions of genes. Alternatively, HDR uses a long stretch of homologous donor DNA, provided endogenously or exogenously, to repair the DSB with high fidelity. HDR is active only in dividing cells, and occurs at a relatively low frequency in most cell types. In many embodiments of the present disclosure, NHEJ is utilized as the repair operant.

[0239] In some embodiments, the Cas9 (CRISPR associated protein 9) endonuclease is from *Streptococcus pyogenes*, although other Cas9 homologs may be used. It should be understood, that wild-type Cas9 may be used or modified versions of Cas9 may be used (e.g., evolved versions of Cas9, or Cas9 orthologues or variants), as provided herein. In some embodiments, Cas9 may be substituted with another RNA-guided endonuclease, such as Cpf1 (of a class II CRISPR/Cas system).

[0240] In some embodiments, the CRISPR/Cas system comprise components derived from a Type-I, Type-II, or Type-III system. Updated classification schemes for CRISPR/Cas loci define Class 1 and Class 2 CRISPR/Cas systems, having Types I to V or VI (Makarova et al., (2015) Nat Rev Microbiol, 13(11):722-36; Shmakov et al., (2015) Mol Cell, 60:385-397). Class 2 CRISPR/Cas systems have single protein effectors. Cas proteins of Types II, V, and VI are single-protein, RNA-guided endonucleases, herein called "Class 2 Cas nucleases." Class 2 Cas nucleases include, for example, Cas9, Cpf1, C2c1, C2c2, and C2c3 proteins. The Cpf1 nuclease (Zetsche et al., (2015) Cell 163:1-13) is homologous to Cas9, and contains a RuvC-like nuclease domain.

[0241] In some embodiments, the Cas nuclease is from a Type-II CRISPR/Cas system (e.g., a Cas9 protein from a CRISPR/Cas9 system). In some embodiments, the Cas nuclease is from a Class 2 CRISPR/Cas system (a single-protein Cas nuclease such as a Cas9 protein or a Cpf1 protein). The Cas9 and Cpf1 family of proteins are enzymes with DNA endonuclease activity, and they can be directed to cleave a desired nucleic acid target by designing an appropriate guide RNA, as described further herein.

[0242] In some embodiments, a Cas nuclease may comprise more than one nuclease domain. For example, a Cas9 nuclease may comprise at least one RuvC-like nuclease domain (e.g. Cpf1) and at least one HNH-like nuclease domain (e.g. Cas9). In some embodiments, the Cas9 nuclease introduces a DSB in the target sequence. In some embodiments, the Cas9 nuclease is modified to contain only one functional nuclease domain. For example, the Cas9 nuclease is modified such that one of the nuclease domains is mutated or fully or partially deleted to reduce its nucleic acid cleavage activity. In some embodiments, the Cas9 nuclease is modified to contain no functional RuvC-like nuclease domain. In other embodiments, the Cas9 nuclease is modified to contain no functional HNH-like nuclease domain. In some embodiments in which only one of the nuclease domains is functional, the Cas9 nuclease is a nickase that is capable of introducing a single-stranded break (a "nick") into the target sequence. In some embodiments, a conserved amino acid within a Cas9 nuclease nuclease domain is substituted to reduce or alter a nuclease activity. In some embodiments, the Cas nuclease nickase comprises an amino acid substitution in the RuvC-like nuclease domain. Exemplary amino acid substitutions in the RuvC-like nuclease domain include D10A (based on the *S. pyogenes* Cas9 nuclease). In some embodiments, the nickase comprises an amino acid substitution in the HNH-like nuclease domain. Exemplary amino acid substitutions in the HNH-like nuclease domain include E762A, H840A, N863A, H983A, and D986A (based on the *S. pyogenes* Cas9 nuclease).

[0243] In some embodiments, the Cas nuclease is from a Type-I CRISPR/Cas system. In some embodiments, the Cas nuclease is a component of the Cascade complex of a Type-I CRISPR/Cas system. For example, the Cas nuclease is a Cas3 nuclease. In some embodiments, the Cas nuclease is derived from a Type-III CRISPR/Cas system. In some embodiments, the Cas nuclease is derived from Type-IV CRISPR/Cas system. In some embodiments, the Cas nuclease is derived from a Type-V CRISPR/Cas system. In some embodiments, the Cas nuclease is derived from a Type-VI CRISPR/Cas system.

Guide RNAs

[0244] The present disclosure provides a genome-targeting nucleic acid that can direct the activities of an associated polypeptide (e.g., a site-directed polypeptide) to a specific target sequence within a target nucleic acid. The genome-targeting nucleic acid can be an RNA. A genome-targeting RNA is referred to as a "guide RNA" or "gRNA" herein. A guide RNA comprises at least a spacer sequence that hybridizes to a target nucleic acid sequence of interest, and a CRISPR repeat sequence. In Type II systems, the gRNA also comprises a second RNA called the tracrRNA sequence. In the Type II gRNA, the CRISPR repeat sequence and tracrRNA sequence hybridize to each other to form a duplex. In the Type V gRNA, the crRNA forms a duplex. In both systems, the duplex binds a site-directed polypeptide, such that the guide RNA and site-direct polypeptide form a complex. In some embodiments, the genome-targeting nucleic acid provides target specificity to the complex by virtue of its association with the site-directed polypeptide. The genome-targeting nucleic acid thus directs the activity of the site-directed polypeptide.

[0245] As is understood by the person of ordinary skill in the art, each guide RNA is designed to include a spacer sequence complementary to its genomic target sequence. See Jinek et al., Science, 337, 816-821 (2012) and Deltcheva et al., Nature, 471, 602-607 (2011).

[0246] In some embodiments, the genome-targeting nucleic acid (e.g., gRNA) is a double-molecule guide RNA. In some embodiments, the genome-targeting nucleic acid (e.g., gRNA) is a single-molecule guide RNA.

[0247] A double-molecule guide RNA comprises two strands of RNA. The first strand comprises in the 5′ to 3′ direction, an optional spacer extension sequence, a spacer sequence and a minimum CRISPR repeat sequence. The second strand comprises a minimum tracrRNA sequence (complementary to the minimum CRISPR repeat sequence), a 3′ tracrRNA sequence and an optional tracrRNA extension sequence.

[0248] A single-molecule guide RNA (referred to as a "sgRNA") in a Type II system comprises, in the 5′ to 3′ direction, an optional spacer extension sequence, a spacer sequence, a minimum CRISPR repeat sequence, a single-molecule guide linker, a minimum tracrRNA sequence, a 3′ tracrRNA sequence and an optional tracrRNA extension sequence. The optional tracrRNA extension may comprise elements that contribute additional functionality (e.g., stability) to the guide RNA. The single-molecule guide linker links the minimum CRISPR repeat and the minimum tracrRNA sequence to form a hairpin structure. The optional tracrRNA extension comprises one or more hairpins.

[0249] A single-molecule guide RNA in a Type V system comprises, in the 5′ to 3′ direction, a minimum CRISPR repeat sequence and a spacer sequence.

[0250] In some embodiments, the sgRNA comprises a 20 nucleotide spacer sequence at the 5′ end of the sgRNA sequence. In some embodiments, the sgRNA comprises a less than 20 nucleotide spacer sequence at the 5′ end of the sgRNA sequence. In some embodiments, the sgRNA comprises a more than 20 nucleotide spacer sequence at the 5′ end of the sgRNA sequence. In some embodiments, the sgRNA comprises a variable length spacer sequence with 17-30 nucleotides at the 5′ end of the sgRNA sequence (see Table 3).

[0251] In some embodiments, the sgRNA comprises comprise no uracil at the 3′ end of the sgRNA sequence. In some embodiments, the sgRNA comprises comprise one or more uracil at the 3′ end of the sgRNA sequence. For example, the sgRNA can comprise 1 uracil (U) at the 3′ end of the

sgRNA sequence. The sgRNA can comprise 2 uracil (UU) at the 3′ end of the sgRNA sequence. The sgRNA can comprise 3 uracil (UUU) at the 3′ end of the sgRNA sequence. The sgRNA can comprise 4 uracil (UUUU) at the 3′ end of the sgRNA sequence. The sgRNA can comprise 5 uracil (UUUUU) at the 3′ end of the sgRNA sequence. The sgRNA can comprise 6 uracil (UUUUUU) at the 3′ end of the sgRNA sequence. The sgRNA can comprise 7 uracil (UUUUUUU) at the 3′ end of the sgRNA sequence. The sgRNA can comprise 8 uracil (UUUUUUUU) at the 3′ end of the sgRNA sequence.

[0252] The sgRNA can be unmodified or modified. For example, modified sgRNAs can comprise one or more 2′-O-methyl phosphorothioate nucleotides.

TABLE-US-00003 TABLE 3 SEQ ID NO. sgRNA sequence 15 nnnnnnnnnnnnnnnnnnnnnguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccga gucggugcuuuu 16 nnnnnnnnnnnnnnnnnnnnnguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacuugaaaaaguggcaccga gucggugc 17 n.sub.(17-30)guuuuagagcuagaaauagcaaguuaaaauaaggcuagu ccguuaucaacuugaaaaaguggcaccgagucggugcu.sub.(1-8)

[0253] By way of illustration, guide RNAs used in the CRISPR/Cas/Cpf1 system, or other smaller RNAs can be readily synthesized by chemical means, as illustrated below and described in the art. While chemical synthetic procedures are continually expanding, purifications of such RNAs by procedures such as high performance liquid chromatography (HPLC, which avoids the use of gels such as PAGE) tends to become more challenging as polynucleotide lengths increase significantly beyond a hundred or so nucleotides. One approach used for generating RNAs of greater length is to produce two or more molecules that are ligated together. Much longer RNAs, such as those encoding a Cas9 or Cpf1 endonuclease, are more readily generated enzymatically.

[0254] Various types of RNA modifications can be introduced during or after chemical synthesis and/or enzymatic generation of RNAs, e.g., modifications that enhance stability, reduce the likelihood or degree of innate immune response, and/or enhance other attributes, as described in the art.

[0255] In some embodiments, indel frequency (editing frequency) may be determined using a TIDE analysis, which can be used to identify highly efficient gRNA molecules. In some embodiments, a highly efficient gRNA yields a gene editing frequency of higher than 80%. For example, a gRNA is considered to be highly efficient if it yields a gene editing frequency of at least 80%, at least 85%, at least 90%, at least 95%, or 100%.

[0256] In some embodiments, gene disruption may occur by deletion of a genomic sequence using two guide RNAs. Methods of using CRISPR-Cas gene editing technology to create a genomic deletion in a cell (e.g., to knock out a gene in a cell) are known (Bauer D E et al. *Vis. Exp.* 2015; 95; e52118).

Spacer Sequence

[0257] In some embodiments, a gRNA comprises a spacer sequence. A spacer sequence is a sequence (e.g., a 20 nucleotide sequence) that defines the target sequence (e.g., a DNA target sequences, such as a genomic target sequence) of a target nucleic acid of interest. In some embodiments, the spacer sequence is 15 to 30 nucleotides. In some embodiments, the spacer sequence is 15, 16, 17, 18, 19, 29, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. In some embodiments, a spacer sequence is 20 nucleotides.

[0258] The "target sequence" is adjacent to a PAM sequence and is the sequence modified by an RNA-guided nuclease (e.g., Cas9). The "target nucleic acid" is a double-stranded molecule: one strand comprises the target sequence and is referred to as the "PAM strand," and the other complementary strand is referred to as the "non-PAM strand." One of skill in the art recognizes that the gRNA spacer sequence hybridizes to the reverse complement of the target sequence, which is located in the non-PAM strand of the target nucleic acid of interest. Thus, the gRNA spacer

sequence is the RNA equivalent of the target sequence. For example, if the target sequence is 5′-AGAGCAACAGTGCTGTGGCC-3′ (SEQ ID NO: 86), then the gRNA spacer sequence is 5′-AGAGCAACAGUGCUGUGGCC-3′ (SEQ ID NO: 98). The spacer of a gRNA interacts with a target nucleic acid of interest in a sequence-specific manner via hybridization (i.e., base pairing). The nucleotide sequence of the spacer thus varies depending on the target sequence of the target nucleic acid of interest.

[0259] In a CRISPR/Cas system herein, the spacer sequence is designed to hybridize to a region of the target nucleic acid that is located 5′ of a PAM of the Cas9 enzyme used in the system. The spacer may perfectly match the target sequence or may have mismatches. Each Cas9 enzyme has a particular PAM sequence that it recognizes in a target DNA. For example, *S. pyogenes* recognizes in a target nucleic acid a PAM that comprises the sequence 5′-NRG-3′, where R comprises either A or G, where N is any nucleotide and N is immediately 3′ of the target nucleic acid sequence targeted by the spacer sequence.

[0260] In some embodiments, the target nucleic acid sequence comprises 20 nucleotides. In some embodiments, the target nucleic acid comprises less than 20 nucleotides. In some embodiments, the target nucleic acid comprises more than 20 nucleotides. In some embodiments, the target nucleic acid comprises at least: 5, 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 or more nucleotides. In some embodiments, the target nucleic acid comprises at most: 5, 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 or more nucleotides. In some embodiments, the target nucleic acid sequence comprises 20 bases immediately 5′ of the first nucleotide of the PAM. For example, in a sequence comprising 5′-NNNNNNNNNNNNNNNNNNNNNRG-3′, the target nucleic acid comprises the sequence that corresponds to the Ns, wherein N is any nucleotide, and the underlined NRG sequence is the *S. pyogenes* PAM.

[0261] Non-limiting examples of gRNAs that may be used as provided herein are provided in PCT/IB2018/001619, filed May 11, 2018, herein incorporated by this reference.

Methods of Making gRNAs

[0262] The gRNAs of the present disclosure are produced by a suitable means available in the art, including but not limited to in vitro transcription (IVT), synthetic and/or chemical synthesis methods, or a combination thereof. Enzymatic (IVT), solid-phase, liquid-phase, combined synthetic methods, small region synthesis, and ligation methods are utilized. In one embodiment, the gRNAs are made using IVT enzymatic synthesis methods. Methods of making polynucleotides by IVT are known in the art and are described in International Application PCT/US2013/30062. Accordingly, the present disclosure also includes polynucleotides, e.g., DNA, constructs and vectors are used to in vitro transcribe a gRNA described herein.

[0263] In some embodiments, non-natural modified nucleobases are introduced into polynucleotides, e.g., gRNA, during synthesis or post-synthesis. In certain embodiments, modifications are on internucleoside linkages, purine or pyrimidine bases, or sugar. In some embodiments, a modification is introduced at the terminal of a polynucleotide; with chemical synthesis or with a polymerase enzyme. Examples of modified nucleic acids and their synthesis are disclosed in PCT application No. PCT/US2012/058519. Synthesis of modified polynucleotides is also described in Verma and Eckstein, Annual Review of Biochemistry, vol. 76, 99-134 (1998).

[0264] In some embodiments, enzymatic or chemical ligation methods are used to conjugate polynucleotides or their regions with different functional moieties, such as targeting or delivery agents, fluorescent labels, liquids, nanoparticles, etc. Conjugates of polynucleotides and modified polynucleotides are reviewed in Goodchild, Bioconjugate Chemistry, vol. 1(3), 165-187 (1990).

[0265] Certain embodiments of the invention also provide nucleic acids, e.g., vectors, encoding gRNAs described herein. In some embodiments, the nucleic acid is a DNA molecule. In other embodiments, the nucleic acid is an RNA molecule. In some embodiments, the nucleic acid comprises a nucleotide sequence encoding a crRNA. In some embodiments, the nucleotide sequence encoding the crRNA comprises a spacer flanked by all or a portion of a repeat sequence

from a naturally-occurring CRISPR/Cas system. In some embodiments, the nucleic acid comprises a nucleotide sequence encoding a tracrRNA. In some embodiments, the crRNA and the tracrRNA is encoded by two separate nucleic acids. In other embodiments, the crRNA and the tracrRNA is encoded by a single nucleic acid. In some embodiments, the crRNA and the tracrRNA is encoded by opposite strands of a single nucleic acid. In other embodiments, the crRNA and the tracrRNA is encoded by the same strand of a single nucleic acid.

[0266] In some embodiments, the gRNAs provided by the disclosure are chemically synthesized by any means described in the art (see e.g., WO/2005/01248). While chemical synthetic procedures are continually expanding, purifications of such RNAs by procedures such as high performance liquid chromatography (HPLC, which avoids the use of gels such as PAGE) tends to become more challenging as polynucleotide lengths increase significantly beyond a hundred or so nucleotides. One approach used for generating RNAs of greater length is to produce two or more molecules that are ligated together.

[0267] In some embodiments, the gRNAs provided by the disclosure are synthesized by enzymatic methods (e.g., in vitro transcription, IVT).

[0268] Various types of RNA modifications can be introduced during or after chemical synthesis and/or enzymatic generation of RNAs, e.g., modifications that enhance stability, reduce the likelihood or degree of innate immune response, and/or enhance other attributes, as described in the art.

[0269] In certain embodiments, more than one guide RNA can be used with a CRISPR/Cas nuclease system. Each guide RNA may contain a different targeting sequence, such that the CRISPR/Cas system cleaves more than one target nucleic acid. In some embodiments, one or more guide RNAs may have the same or differing properties such as activity or stability within the Cas9 RNP complex. Where more than one guide RNA is used, each guide RNA can be encoded on the same or on different vectors. The promoters used to drive expression of the more than one guide RNA is the same or different.

[0270] The guide RNA may target any sequence of interest via the targeting sequence (e.g., spacer sequence) of the crRNA. In some embodiments, the degree of complementarity between the targeting sequence of the guide RNA and the target sequence on the target nucleic acid molecule is about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100%. In some embodiments, the targeting sequence of the guide RNA and the target sequence on the target nucleic acid molecule is 100% complementary. In other embodiments, the targeting sequence of the guide RNA and the target sequence on the target nucleic acid molecule may contain at least one mismatch. For example, the targeting sequence of the guide RNA and the target sequence on the target nucleic acid molecule may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 mismatches. In some embodiments, the targeting sequence of the guide RNA and the target sequence on the target nucleic acid molecule may contain 1-6 mismatches. In some embodiments, the targeting sequence of the guide RNA and the target sequence on the target nucleic acid molecule may contain 5 or 6 mismatches.

[0271] The length of the targeting sequence may depend on the CRISPR/Cas9 system and components used. For example, different Cas9 proteins from different bacterial species have varying optimal targeting sequence lengths. Accordingly, the targeting sequence may comprise 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, 35, 40, 45, 50, or more than 50 nucleotides in length. In some embodiments, the targeting sequence may comprise 18-24 nucleotides in length. In some embodiments, the targeting sequence may comprise 19-21 nucleotides in length. In some embodiments, the targeting sequence may comprise 20 nucleotides in length.

[0272] In some embodiments of the present disclosure, a CRISPR/Cas nuclease system includes at least one guide RNA. In some embodiments, the guide RNA and the Cas protein may form a ribonucleoprotein (RNP), e.g., a CRISPR/Cas complex. The guide RNA may guide the Cas protein

to a target sequence on a target nucleic acid molecule (e.g., a genomic DNA molecule), where the the Cas protein cleaves the target nucleic acid. In some embodiments, the CRISPR/Cas complex is a Cpf1/guide RNA complex. In some embodiments, the CRISPR complex is a Type-II CRISPR/Cas9 complex. In some embodiments, the Cas protein is a Cas9 protein. In some embodiments, the CRISPR/Cas9 complex is a Cas9/guide RNA complex.

Delivery of Guide RNA and Nuclease

[0273] In some embodiments, a gRNA and an RNA-guided nuclease are delivered to a cell separately, either simultaneously or sequentially. In some embodiments, a gRNA and an RNA-guided nuclease are delivered to a cell together. In some embodiments, a gRNA and an RNA-guided nuclease are pre-complexed together to form a ribonucleoprotein (RNP).

[0274] RNPs are useful for gene editing, at least because they minimize the risk of promiscuous interactions in a nucleic acid-rich cellular environment and protect the RNA from degradation. Methods for forming RNPs are known in the art. In some embodiments, an RNP containing an RNA-guided nuclease (e.g., a Cas nuclease, such as a Cas9 nuclease) and a gRNA targeting a gene of interest is delivered a cell (e.g.: a T cell). In some embodiments, an RNP is delivered to a T cell by electroporation.

[0275] As used herein, a "TRAC targeting RNP" refers to a gRNA that targets the TRAC gene pre-complexed with an RNA-guided nuclease. As used herein, a "β2M targeting RNP" refers to a gRNA that targets the β2M gene pre-complexed with an RNA-guided nuclease. As used herein, a "CD70 targeting RNP" refers to a gRNA that targets the CD70 gene pre-complexed with an RNA-guided nuclease. As used herein, a "PD-1 targeting RNP" refers to a gRNA that targets the PD-1 gene pre-complexed with an RNA-guided nuclease.

[0276] In some embodiments, a TRAC targeting RNP is delivered to a cell. In some embodiments, a β2M targeting RNP is delivered to a cell. In some embodiments, a CD70 targeting RNP is delivered to a cell. In some embodiments, a PD-1 targeting RNP is delivered to a cell.

[0277] In some embodiments, more than one RNP is delivered to a cell. In some embodiments, more than on RNP is delivered to a cell separately. In some embodiments, more than one RNP is delivered to a cell simultaneously. In some embodiments, at least one of the following RNPs is delivered to a cell: [0278] (i) a TRAC targeting RNP; [0279] (ii) a β2M targeting RNP; [0280] (iii) a CD70 targeting RNP; or [0281] (iv) a PD-1 targeting RNP. In some embodiments, at least two of the following RNPs are delivered to a cell: [0282] (i) a TRAC targeting RNP; [0283] (ii) a β2M targeting RNP; [0284] (iii) a CD70 targeting RNP; or [0285] (iv) a PD-1 targeting RNP.

[0286] In some embodiments, an RNA-guided nuclease is delivered to a cell in a DNA vector that expresses the RNA-guided nuclease, an RNA that encodes the RNA-guided nuclease, or a protein. In some embodiments, a gRNA targeting a gene is delivered to a cell as an RNA, or a DNA vector that expresses the gRNA.

[0287] Delivery of an RNA-guided nuclease, gRNA, and/or an RNP may be through direct injection or cell transfection using known methods, for example, electroporation or chemical transfection. Other cell transfection methods may be used.

Chimeric Antigen Receptor (CAR) T Cells

[0288] A chimeric antigen receptor refers to an artificial immune cell receptor that is engineered to recognize and bind to an antigen expressed by tumor cells. Generally, a CAR is designed for a T cell and is a chimera of a signaling domain of the T-cell receptor (TCR) complex and an antigen-recognizing domain (e.g., a single chain fragment (scFv) of an antibody or other antibody fragment) (Enblad et al., Human Gene Therapy. 2015; 26(8):498-505). A T cell that expresses a CAR is referred to as a CAR T cell. CARs have the ability to redirect T-cell specificity and reactivity toward a selected target in a non-MHC-restricted manner. The non-MHC-restricted antigen recognition gives T-cells expressing CARs the ability to recognize an antigen independent of antigen processing, thus bypassing a major mechanism of tumor escape. Moreover, when expressed in T-cells, CARs advantageously do not dimerize with endogenous T-cell receptor (TCR)

alpha and beta chains. CARs are often referenced to by the antigen they bind. For example, a "CD19 CAR", a "CD70 CAR", a "CD33 CAR" and a "BCMA CAR" are CARs comprising antigen binding domains that specifically bind to CD19, CD70, CD33 or BCMA, respectively. Accordingly, such terms are interchangeable with anti-CD19 CAR, anti-CD70 CAR, anti-CD33 CAR and anti-BCMA CAR. It will be understood by those of ordinary skill in the art that a CAR that specifically binds an antigen can be referred to with either terminology.

[0289] There are four generations of CARs, each of which contains different components. First generation CARs join an antibody-derived scFv to the CD3zeta ((or z) intracellular signaling domain of the T-cell receptor through hinge and transmembrane domains. Second generation CARs incorporate an additional domain, e.g., CD28, 4-1BB (41BB), or ICOS, to supply a costimulatory signal. Third-generation CARs contain two costimulatory domains fused with the TCR CD3ζ chain. Third-generation costimulatory domains may include, e.g., a combination of CD3ζ, CD27, CD28, 4-1BB, ICOS, or OX40. CARs, in some embodiments, contain an ectodomain, commonly derived from a single chain variable fragment (scFv), a hinge, a transmembrane domain, and an endodomain with one (first generation), two (second generation), or three (third generation) signaling domains derived from CD3Z and/or co-stimulatory molecules (Maude et al., Blood. 2015; 125(26):4017-4023; Kakarla and Gottschalk, *Cancer J.* 2014; 20(2):151-155).

[0290] CARs typically differ in their functional properties. The CD3ζ signaling domain of the T-cell receptor, when engaged, will activate and induce proliferation of T-cells but can lead to anergy (a lack of reaction by the body's defense mechanisms, resulting in direct induction of peripheral lymphocyte tolerance). Lymphocytes are considered anergic when they fail to respond to a specific antigen. The addition of a costimulatory domain in second-generation CARs improved replicative capacity and persistence of modified T-cells. Similar antitumor effects are observed in vitro with CD28 or 4-1BB CARs, but preclinical in vivo studies suggest that 4-1BB CARs may produce superior proliferation and/or persistence. Clinical trials suggest that both of these second-generation CARs are capable of inducing substantial T-cell proliferation in vivo, but CARs containing the 4-1BB costimulatory domain appear to persist longer. Third generation CARs combine multiple signaling domains (costimulatory) to augment potency.

[0291] In some embodiments, a chimeric antigen receptor is a first generation CAR. In other embodiments, a chimeric antigen receptor is a second generation CAR. In yet other embodiments, a chimeric antigen receptor is a third generation CAR.

[0292] A CAR, in some embodiments, comprises an extracellular (ecto) domain comprising an antigen binding domain (e.g., an antibody, such as an scFv), a transmembrane domain, and a cytoplasmic (endo) domain.

Ectodomain

[0293] The ectodomain is the region of the CAR that is exposed to the extracellular fluid and, in some embodiments, includes an antigen binding domain, and optionally a signal peptide, a spacer domain, and/or a hinge domain. In some embodiments, the antigen binding domain is a single-chain variable fragment (scFv) that includes the VL and VH of immunoglobulins connected with a short linker peptide. The linker, in some embodiments, includes hydrophilic residues with stretches of glycine and serine for flexibility as well as stretches of glutamate and lysine for added solubility. A single-chain variable fragment (scFv) is not actually a fragment of an antibody, but instead is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. This protein retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of the linker. In some embodiments, the scFv of the present disclosure is humanized. In other embodiments, the scFv is fully human. In yet other embodiments, the scFv is a chimera (e.g., of mouse and human sequence).

[0294] In some embodiments, the scFv is an anti-CD70 scFv (binds specifically to CD70). Non-limiting examples of anti-CD70 scFv proteins that may be used as provided herein may include the amino acid sequence of SEQ ID NO: 48 or SEQ ID NO: 50.

[0295] In some embodiments, the scFv is an anti-BCMA scFv (binds specifically to BCMA). Non-limiting examples of anti-BCMA scFv proteins that may be used as provided herein may include the amino acid sequence of SEQ ID NO: 59.

[0296] In some embodiments, the scFv is an anti-CD19 scFv (binds specifically to CD19). Non-limiting examples of anti-CD19 scFv proteins that may be used as provided herein may include the amino acid sequence of SEQ ID NO: 151.

[0297] In some embodiments, the scFv is an anti-CD33 scFv (binds specifically to CD33). Non-limiting examples of anti-CD33 scFv proteins that may be used as provided herein may include the amino acid sequence of SEQ ID NO: 137.

[0298] Other scFv proteins may be used.

[0299] The signal peptide can enhance the antigen specificity of CAR binding. Signal peptides can be derived from antibodies, such as, but not limited to, CD8, as well as epitope tags such as, but not limited to, GST or FLAG. Examples of signal peptides include MLLLVTSLLLCELPHPAFLLIP (SEQ ID NO: 88) and MALPVTALLLPLALLLHAARP (SEQ ID NO: 89). Other signal peptides may be used.

[0300] In some embodiments, a spacer domain or hinge domain is located between an extracellular domain (comprising the antigen binding domain) and a transmembrane domain of a CAR, or between a cytoplasmic domain and a transmembrane domain of the CAR. A spacer domain is any oligopeptide or polypeptide that functions to link the transmembrane domain to the extracellular domain and/or the cytoplasmic domain in the polypeptide chain. A hinge domain is any oligopeptide or polypeptide that functions to provide flexibility to the CAR, or domains thereof, or to prevent steric hindrance of the CAR, or domains thereof. In some embodiments, a spacer domain or a hinge domain may comprise up to 300 amino acids (e.g., 10 to 100 amino acids, or 5 to 20 amino acids). In some embodiments, one or more spacer domain(s) may be included in other regions of a CAR. In some embodiments, the hinge domain is a CD8 hinge domain. Other hinge domains may be used.

Transmembrane Domain

[0301] The transmembrane domain is a hydrophobic alpha helix that spans the membrane. The transmembrane domain provides stability of the CAR. In some embodiments, the transmembrane domain of a CAR as provided herein is a CD8 transmembrane domain. In other embodiments, the transmembrane domain is a CD28 transmembrane domain. In yet other embodiments, the transmembrane domain is a chimera of a CD8 and CD28 transmembrane domain. Other transmembrane domains may be used as provided herein. In some embodiments, the transmembrane domain is a CD8a transmembrane domain:
FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNR (SEQ ID NO: 90). Other transmembrane domains may be used.

[0302] In some embodiments, the transmembrane domain is a CD8a transmembrane domain comprising the amino acid sequence: IYIWAPLAGTCGVLLLSLVITLY (SEQ ID NO: 126).

Endodomain

[0303] The endodomain is the functional end of the receptor. Following antigen recognition, receptors cluster and a signal is transmitted to the cell. The most commonly used endodomain component is CD3-zeta, which contains three (3) immunoreceptor tyrosine-based activation motif (ITAM)s. This transmits an activation signal to the T cell after the antigen is bound. In many cases, CD3-zeta may not provide a fully competent activation signal and, thus, a co-stimulatory signaling is used. For example, CD28 and/or 4-1BB may be used with CD3-zeta (CD3ζ) to transmit a proliferative/survival signal. Thus, in some embodiments, the co-stimulatory molecule of a CAR as

provided herein is a CD28 co-stimulatory molecule. In other embodiments, the co-stimulatory molecule is a 4-1BB co-stimulatory molecule. In some embodiments, a CAR includes CD3ζ and CD28. In other embodiments, a CAR includes CD3-zeta and 4-1BB. In still other embodiments, a CAR includes CD3ζ, CD28, and 4-1BB. Table 4 provides examples of signaling domains derived from 4-1BB, CD28 and CD3-zeta that may be used herein.

TABLE-US-00004 TABLE 4

SEQ ID	Name	Sequence NO: 4-
AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAAC AACCATTTATGAGACCAGTACAAACTACTCAAGAGGA AGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAA GGAGGATGTGAACTG	18	1BB
KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEE	19	GGCEL CD28
TCAAAGCGGAGTAGGTTGTTGCATTCCGATTACATGA ATATGACTCCTCGCCGGCCTGGGCCGACAAGAAAACA TTACCAACCCTATGCCCCCCCACGAGACTTCGCTGCG TACAGGTCC	121	
SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAA	20	YRS CD3-
CGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGGCAT ATCAGCAAGGACAGAATCAGCTGTATAACGAACTGAA TTTGGGACGCCGCGAGGAGTATGACGTGCTTGATAAA CGCCGGGGGGAGAGACCCGGAAATGGGGGGGTAAACCCC GAAGAAAGAATCCCCAAGAAGGACTCTACAATGAACT CCAGAAGGATAAGATGGCGGAGGCCTACTCAGAAATA GGTATGAAGGGCGAACGACGACGGGGAAAAGGTCACG ATGGCCTCTACCAAGGGTTGAGTACGGCAACCAAAGA TACGTACGATGCACTGCATATGCAGGCCCTGCCTCCC AGA	21	zeta
RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPP R	22	

Cancer Antigens

CD70

[0304] In some embodiments, the T cells of the present disclosure are engineered with a chimeric antigen receptor (CAR) designed to target CD70. CD70 was initially identified as the ligand for CD27, a co-stimulatory receptor involved in T cell proliferation and survival. CD70 is only found on a small percentage of activated T cells and antigen presenting cells in draining lymph nodes during viral infection. Many human tumors also express CD70 including, but not limited to, solid cancers such as clear cell renal cancer, breast cancer, gastric cancer, ovarian cancer, glioblastoma, and hematological malignancies. Due to its restricted expression pattern on normal tissues and overexpression in numerous cancers, CD70 is an attractive therapeutic target.

[0305] Thus, in some embodiments, T cells of the present disclosure are engineered to express a CAR comprising an anti-CD70 antibody (e.g., anti-CD70 scFv). In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv encoded by the sequence of SEQ ID NO: 47 or 49. In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv comprising the sequence of SEQ ID NO: 48 or 50. In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv comprising a VH comprising the sequence of SEQ ID NO: 51. In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv comprising a VL comprising the sequence of SEQ ID NO: 52. In some embodiments, a CAR comprising an anti-CD70 antibody is encoded by the sequence of SEQ ID NO: 45. In some embodiments, a CAR comprising an anti-CD70 antibody comprises the sequence of SEQ ID NO: 46.

[0306] In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 47 or 49. In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv comprising an amino acid sequence

having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 48 or 50. In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv comprising a VH comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 51. In some embodiments, the anti-CD70 antibody is an anti-CD70 scFv comprising a VL comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 52. In some embodiments, a CAR comprising an anti-CD70 antibody is encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 45. In some embodiments, a CAR comprising an anti-CD70 antibody comprises an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 46.

BCMA

[0307] In some embodiments, the T cells of the present disclosure are engineered with a CAR designed to target BCMA. B-cell maturation antigen (BCMA, CD269) is a member of the tumor necrosis factor receptor (TNF) superfamily. BCMA binds B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL). Among nonmalignant cells, BCMA is expressed primarily by plasma cells and subsets of mature B cells. BCMA is selectively expressed by B-lineage cells including multiple myeloma cells and non-Hodgkin's lymphoma, thus BCMA is also an attractive therapeutic target.

[0308] Thus, in some embodiments, T cells of the present disclosure are engineered to express a CAR comprising an anti-BCMA antibody (e.g., anti-BCMA scFv). In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv encoded by the sequence of SEQ ID NO: 58. In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv comprising the sequence of SEQ ID NO: 59. In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv comprising a VH comprising the sequence of SEQ ID NO: 60. In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv comprising a VL comprising the sequence of SEQ ID NO: 61. In some embodiments, a CAR comprising an anti-BCMA antibody is encoded by the sequence of SEQ ID NO: 56. In some embodiments, a CAR comprising an anti-BCMA antibody comprises the sequence of SEQ ID NO: 57.

[0309] In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 58. In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 59. In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv comprising a VH comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 60. In some embodiments, the anti-BCMA antibody is an anti-BCMA scFv comprising a VL comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 61. In some embodiments, a CAR comprising an anti-BCMA antibody is encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 56. In some embodiments, a CAR comprising an anti-BCMA antibody comprises an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 57.

CD19

[0310] In some embodiments, the T cells of the present disclosure are engineered with a CAR designed to target CD19. Cluster of Differentiation 19 (CD19) is an antigenic determinant detectable on leukemia precursor cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequence of human CD19 can be found as UniProt/Swiss-Prot Accession No. P15391 and the nucleotide sequence encoding of the human CD19 can be found at Accession No. NM-001178098. CD19 is expressed on most B lineage cancers, including, e.g., acute lymphoblastic leukemia, chronic lymphocyte leukemia and non-Hodgkin's lymphoma. It is also an early marker of B cell progenitors. See, e.g., Nicholson et al. Mol. Immun. 34 (16-17): 1157-1165 (1997).

[0311] Thus, in some embodiments, T cells of the present disclosure are engineered to express a CAR comprising an anti-CD19 antibody (e.g., anti-CD19 scFv). In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv encoded by the sequence of SEQ ID NO: 150. In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv comprising the sequence of SEQ ID NO: 151. In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv comprising a VH comprising the sequence of SEQ ID NO: 152. In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv comprising a VL comprising the sequence of SEQ ID NO: 153. In some embodiments, a CAR comprising an anti-CD19 antibody is encoded by the sequence of SEQ ID NO: 148. In some embodiments, a CAR comprising an anti-CD19 antibody comprises the sequence of SEQ ID NO: 149.

[0312] In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 150. In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 151. In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv comprising a VH comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 152. In some embodiments, the anti-CD19 antibody is an anti-CD19 scFv comprising a VL comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 153. In some embodiments, a CAR comprising an anti-CD19 antibody is encoded by a nucleotide having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 148. In some embodiments, a CAR comprising an anti-CD19 antibody comprises an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 149.

CD33

[0313] In some embodiments, the T cells of the present disclosure are engineered with a CAR designed to target CD33. CD33, also known as Siglec3, is a transmembrane receptor expressed on cells of myeloid lineage that is known to bind sialic acids. As CD33 is expressed in cancer cells (e.g., acute myeloid leukemia), it is thought that CD33 represents a cell surface marker for targeting these malignancies.

[0314] Thus, in some embodiments, T cells of the present disclosure are engineered to express a CAR comprising an anti-CD33 antibody (e.g., anti-CD33 scFv). In some embodiments, the anti-CD33 antibody is an anti-CD33 scFv encoded by the sequence of SEQ ID NO: 138. In some embodiments, the anti-CD33 antibody is an anti-CD33 scFv comprising the sequence of SEQ ID NO: 137. In some embodiments, the anti-CD33 antibody is an anti-CD19 scFv comprising a VH comprising the sequence of SEQ ID NO: 140. In some embodiments, the anti-CD33 antibody is an

anti-CD33 scFv comprising a VL comprising the sequence of SEQ ID NO: 141. In some embodiments, a CAR comprising an anti-CD33 antibody is encoded by the sequence of SEQ ID NO: 136. In some embodiments, a CAR comprising an anti-CD33 antibody comprises the sequence of SEQ ID NO: 139.

[0315] In some embodiments, the anti-CD33 antibody is an anti-CD33 scFv encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 138. In some embodiments, the anti-CD33 antibody is an anti-CD33 scFv comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 137. In some embodiments, the anti-CD33 antibody is an anti-CD19 scFv comprising a VH comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 140. In some embodiments, the anti-CD33 antibody is an anti-CD33 scFv comprising a VL comprising an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 141. In some embodiments, a CAR comprising an anti-CD33 antibody is encoded by a nucleotide sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 136. In some embodiments, a CAR comprising an anti-CD33 antibody comprises an amino acid sequence having at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% 98% or 99% identity to SEQ ID NO: 139.

Antibodies

[0316] An antibody (interchangeably used in plural form) is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, etc., through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule. As used herein, the term "antibody" encompasses not only intact (i.e., full-length) monoclonal antibodies, but also antigen-binding fragments (such as Fab, Fab′, F(ab′)2, Fv), single chain variable fragment (scFv), mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, chimeric antibodies, diabodies, linear antibodies, single chain antibodies, single domain antibodies (e.g., camel or llama V.sub.HH antibodies), multispecific antibodies (e.g., bispecific antibodies) and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies.

[0317] A typical antibody molecule comprises a heavy chain variable region (VH) and a light chain variable region (VL), which are usually involved in antigen binding. These regions/residues that are responsible for antigen-binding can be identified from amino acid sequences of the VH/VL sequences of a reference antibody (e.g., an anti-CD70 antibody or an anti-BCMA antibody as described herein) by methods known in the art. The VH and VL regions can be further subdivided into regions of hypervariability, also known as "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, which are known as "framework regions" ("FR"). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The extent of the framework region and CDRs can be precisely identified using methodology known in the art, for example, by the Kabat definition, the Chothia definition, the AbM definition, and/or the contact definition, all of which are well known in the art. As used herein, a CDR may refer to the CDR defined by any method known in the art. Two antibodies having the same CDR means that the two antibodies have the same amino acid sequence of that CDR as determined by the same method. See, e.g., Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, Chothia et al., (1989) Nature 342:877; Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917,

Al-lazikani et al (1997) J. Molec. Biol. 273:927-948; and Almagro, J. Mol. Recognit. 17:132-143 (2004). See also hgmp.mrc.ac.uk and bioinf.org.uk/abs.

[0318] In some embodiments, an antibody is an scFv, such as an anti-CD70 scFv, an anti-BCMA scFv, an anti-CD19 scFv or an anti-CD33 scFv. An antibody includes an antibody of any class, such as IgD, IgE, IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant domain of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0319] The antibodies to be used as provided herein can be murine, rat, human, or any other origin (including chimeric or humanized antibodies). In some examples, the antibody comprises a modified constant region, such as a constant region that is immunologically inert, e.g., does not trigger complement mediated lysis, or does not stimulate antibody-dependent cell mediated cytotoxicity (ADCC).

[0320] In some embodiments, an antibody of the present disclosure is a humanized antibody. Humanized antibodies refer to forms of non-human (e.g., murine) antibodies that are specific chimeric immunoglobulins, immunoglobulin chains, or antigen-binding fragments thereof that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences, but are included to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. A humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Other forms of humanized antibodies have one or more CDRs (one, two, three, four, five, or six) which are altered with respect to the original antibody, which are also termed one or more CDRs "derived from" one or more CDRs from the original antibody. Humanized antibodies may also involve affinity maturation.

[0321] In some embodiments, an antibody of the present disclosure is a chimeric antibody, which can include a heavy constant region and a light constant region from a human antibody. Chimeric antibodies refer to antibodies having a variable region or part of variable region from a first species and a constant region from a second species. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals (e.g., a non-human mammal such as mouse, rabbit, and rat), while the constant portions are homologous to the sequences in antibodies derived from another mammal such as human. In some embodiments, amino acid modifications can be made in the variable region and/or the constant region.

[0322] In some embodiments, an antibody of the present disclosure specifically binds a target antigen, such as human CD70, human BCMA, human CD19 or human CD33. An antibody that "specifically binds" to a target or an epitope is a term well understood in the art, and methods to determine such specific binding are also well known in the art. A molecule is said to exhibit

"specific binding" if it reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular target antigen than it does with alternative targets. An antibody "specifically binds" to a target antigen if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that specifically (or preferentially) binds to a CD70, BCMA, CD19 or CD33 epitope is an antibody that binds this CD70, BCMA, CD19 or CD33 epitope with greater affinity, avidity, more readily, and/or with greater duration than it binds to other CD70, BCMA, CD19 or CD33 epitopes or non-CD70, non-BCMA, non-CD19 or non-CD33 epitopes. It is also understood by reading this definition that, for example, an antibody that specifically binds to a first target antigen may or may not specifically or preferentially bind to a second target antigen. As such, "specific binding" or "preferential binding" does not necessarily require (although it can include) exclusive binding. Generally, but not necessarily, reference to binding means preferential binding.

[0323] In some embodiments, the equilibrium dissociation constant (K.sub.D) between the antibody and CD70 is 100 pM to 1 μM. In some embodiments, the K.sub.D between the antibody and CD70 is 1 nM to 100 nM.

[0324] In some embodiments, the equilibrium dissociation constant (K.sub.D) between the antibody and BCMA is 100 pM to 1 μM. In some embodiments, the K.sub.D between the antibody and BCMA is 1 nM to 100 nM.

[0325] In some embodiments, the equilibrium dissociation constant (K.sub.D) between the antibody and CD19 is 100 pM to 1 μM. In some embodiments, the K.sub.D between the antibody and CD19 is 1 nM to 100 nM.

[0326] In some embodiments, the equilibrium dissociation constant (K.sub.D) between the antibody and CD33 is 100 pM to 1 μM. In some embodiments, the K.sub.D between the antibody and CD33 is 1 nM to 100 nM.

[0327] Also within the scope of the present disclosure are functional variants of any of the exemplary antibodies as disclosed herein. A functional variant may contain one or more amino acid residue variations in the VH and/or VL, or in one or more of the VH CDRs and/or one or more of the VL CDRs as relative to a reference antibody, while retaining substantially similar binding and biological activities (e.g., substantially similar binding affinity, binding specificity, inhibitory activity, anti-tumor activity, or a combination thereof) as the reference antibody.

[0328] In some examples, an antibody disclosed herein comprises a VH CDR1, a VH CDR2, and a VH CDR3, which collectively contains no more than 10 amino acid variations (e.g., no more than 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid variation) as compared with the VH CDR1, VH CDR2, and VH CDR3 of a reference antibody such as Antibody A (VH: SEQ ID NO: 51; VL: SEQ ID NO: 52) or Antibody B (VH: SEQ ID NO: 60; VL: SEQ ID NO: 61). "Collectively" means that the total number of amino acid variations in all of the three VH CDRs is within the defined range. Alternatively or in addition, antibody may comprise a VL CDR1, a VL CDR2, and a VL CDR3, which collectively contains no more than 10 amino acid variations (e.g., no more than 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid variation) as compared with the VL CDR1, VL CDR2, and VL CDR3 of the reference antibody.

[0329] In some examples, an antibody disclosed herein may comprise a VH CDR1, a VH CDR2, and a VH CDR3, at least one of which contains no more than 5 amino acid variations (e.g., no more than 4, 3, 2, or 1 amino acid variation) as the counterpart VH CDR of a reference antibody such as Antibody A (VH: SEQ ID NO: 51; VL: SEQ ID NO: 52) or Antibody B (VH: SEQ ID NO: 60; VL: SEQ ID NO: 61). In specific examples, the antibody comprises a VH CDR3, which contains no more than 5 amino acid variations (e.g., no more than 4, 3, 2, or 1 amino acid variation) as the VH CDR3 of a reference antibody such as Antibody A (VH: SEQ ID NO: 51; VL: SEQ ID NO: 52) or Antibody B (VH: SEQ ID NO: 60; VL: SEQ ID NO: 61). Alternatively or in addition, an antibody may comprise a VL CDR1, a VL CDR2, and a VL CDR3, at least one of which contains no more than 5 amino acid variations (e.g., no more than 4, 3, 2, or 1 amino acid variation)

as the counterpart VL CDR of the reference antibody. In specific examples, the antibody comprises a VL CDR3, which contains no more than 5 amino acid variations (e.g., no more than 4, 3, 2, or 1 amino acid variation) as the VL CDR3 of the reference antibody.

[0330] In some instances, the amino acid residue variations can be conservative amino acid residue substitutions. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g. Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F. M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) A.fwdarw.G, S; (b) R.fwdarw.K, H; (c) N.fwdarw.Q, H; (d) D.fwdarw.E, N; (e) C+S, A; (f) Q.fwdarw.N; (g) E.fwdarw.D, Q; (h) G+A; (i) H.fwdarw.N, Q; (j) I.fwdarw.L, V; (k) L.fwdarw.I, V; (1) K.fwdarw.R, H; (m) M.fwdarw.L, I, Y; (n) F.fwdarw.Y, M, L; (o) P.fwdarw.A; (p) S.fwdarw.T; (q) T.fwdarw.S; (r) W.fwdarw.Y, F; (s) Y.fwdarw.W, F; and (t) V.fwdarw.I, L.

[0331] In some embodiments, an antibody disclosed herein may comprise VH CDRs that collectively are at least 80% (e.g., 85%, 90%, 95%, or 98%) identical to the VH CDRs of a reference antibody such as Antibody A (VH: SEQ ID NO: 51; VL: SEQ ID NO: 52) or Antibody B (VH: SEQ ID NO: 60; VL: SEQ ID NO: 61). Alternatively or in addition, the antibody may comprise VL CDRs that collectively are at least 80% (e.g., 85%, 90%, 95%, or 98%) identical to the VL CDRs of the reference antibody. In some embodiments, an antibody may comprise a VH that is at least 80% (e.g., 85%, 90%, 95%, or 98%) identical to the VH of a reference antibody such as Antibody A (VH: SEQ ID NO: 51; VL: SEQ ID NO: 52) or Antibody B (VH: SEQ ID NO: 60; VL: SEQ ID NO: 61) and/or a VL that is at least 80% (e.g., 85%, 90%, 95%, or 98%) identical to the VL of the reference antibody.

[0332] In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises a VH and a VL comprising the amino acid sequences set forth in SEQ ID NOs: 51 and 52, respectively. In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 51, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 52. In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 51, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 52, wherein the CDRs are determined according to Kabat. In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 51, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 52, wherein the CDRs are determined according to Chothia. In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 51, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 52, wherein the CDRs are determined according to AbM. In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 68, 70 and 72, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 62, 64 and 66. In some embodiments, an anti-CD70 antibody (e.g., anti-CD70 scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 69, 71 and 73, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 63, 65 and 67. In some embodiments, an anti-CD70 antibody is an anti-CD70 scFv comprising the amino acid sequence set forth in SEQ ID NO: 50. In some embodiments, an anti-CD70 antibody is an anti-CD70 scFv encoded by the nucleotide sequence set forth in SEQ ID NO: 49. In some embodiments, an anti-CD70 antibody is an anti-CD70 scFv comprising the amino

acid sequence set forth in SEQ ID NO: 48. In some embodiments, an anti-CD70 antibody is an anti-CD70 scFv encoded by the nucleotide sequence set forth in SEQ ID NO: 47.

[0333] In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises a VH and a VL comprising the amino acid sequences set forth in SEQ ID NOs: 60 and 61, respectively. In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 60, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 61. In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 60, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 61, wherein the CDRs are determined according to Kabat. In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 60, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 61, wherein the CDRs are determined according to Chothia. In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 60, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 61, wherein the CDRs are determined according to AbM. In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 80, 82 and 84, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 74, 76 and 78. In some embodiments, an anti-BCMA antibody (e.g., anti-BCMA scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 81, 83 and 85, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 75, 77 and 79. In some embodiments, an anti-BCMA antibody is an anti-BCMA scFv comprising the amino acid sequence set forth in SEQ ID NO: 59 In some embodiments, an anti-BCMA antibody is an anti-BCMA scFv encoded by the nucleotide sequence set forth in SEQ ID NO: 58.

[0334] In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises a VH and a VL comprising the amino acid sequences set forth in SEQ ID NOs: 152 and 153, respectively. In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 152, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 153. In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 152, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 153, wherein the CDRs are determined according to Kabat. In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 152, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 153, wherein the CDRs are determined according to Chothia. In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 152, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 153, wherein the CDRs are determined according to AbM. In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 169, 170 and 171, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 166, 167 and 168, respectively. In some embodiments, an anti-CD19 antibody (e.g., anti-CD19 scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 175, 176 and 177, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 172, 173 and 174, respectively. In some embodiments, an anti-CD19 antibody is an anti-CD19 scFv comprising the amino acid sequence set forth in SEQ ID NO: 151. In some embodiments, an anti-CD19 antibody is an anti-CD19 scFv encoded by the nucleotide sequence set forth in SEQ ID NO: 150.

[0335] In some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises a VH and a VL comprising the amino acid sequences set forth in SEQ ID NOs: 140 and 141, respectively. In

some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 140, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 141. In some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 140, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 141, wherein the CDRs are determined according to Kabat. In some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 140, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 141, wherein the CDRs are determined according to Chothia. In some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises three CDRs (CDR1, CDR2 and CDR2) of the VH set forth in SEQ ID NO: 140, and three CDRs (CDR1, CDR2 and CDR3) of the VL set forth in SEQ ID NO: 141, wherein the CDRs are determined according to AbM. In some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 142, 143 and 144, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 145, 146 and 147. In some embodiments, an anti-CD33 antibody (e.g., anti-CD33 scFv) comprises heavy chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 178, 179 and 180, respectively, and light chain CDR1, CDR2 and CDR3 sequences set forth in SEQ ID NOs: 145, 146 and 147. In some embodiments, an anti-CD33 antibody is an anti-CD33 scFv comprising the amino acid sequence set forth in SEQ ID NO: 137. In some embodiments, an anti-CD33 antibody is an anti-CD33 scFv encoded by the nucleotide sequence set forth in SEQ ID NO: 138.

Antigen Targeting Chimeric Antigen Receptor Construct

[0336] In some embodiments, the engineered T cells described herein comprise a tumor antigen targeting CAR. In some embodiments, a tumor antigen is a "tumor associated antigen," referring an immunogenic molecule, such as a protein, that is generally expressed at a higher level in tumor cells than in non-tumor cells, in which it may not be expressed at all, or only at low levels. In some embodiments, tumor-associated structures which are recognized by the immune system of the tumor-harboring host are referred to as tumor-associated antigens. In some embodiments, a tumor-associated antigen is a universal tumor antigen if its broadly expressed by most tumors. In some embodiments, tumor-associated antigens are differentiation antigens, mutational antigens, overexpressed cellular antigens or viral antigens. In some embodiments, a tumor antigen is a "tumor specific antigen" or "TSA," referring to an immunogenic molecule, such as a protein, that is unique to a tumor cell. Tumor specific antigens are exclusively expressed in tumor cells. In some embodiments, the tumor antigen is not CD70.

[0337] In some embodiments, the engineered T cells described herein comprise a non-CD70 targeting CAR (e.g., a CAR that does not bind CD70).

CD19 CAR

[0338] In some embodiments, the engineered T cells described herein comprise a CD19 targeting CAR, also referred to herein as CD19 CAR, anti-CD19 CAR or anti-CD19 CAR T cells. In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 antigen-binding domain, (ii) a transmembrane domain, and (iii) an endodomain comprising at least one co-stimulatory domain.

[0339] In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 or 41BB co-stimulatory domain, and a CD3-zeta signaling domain. In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 co-stimulatory domain and a CD3-zeta signaling domain. In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that

comprises a 41BB co-stimulatory domain and a CD3-zeta signaling domain.

[0340] In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 antigen-binding domain, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a CD28 co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 20 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0341] In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 scFv comprising the amino acid sequence set forth in SEQ ID NO: 151, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a CD28 co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 20 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0342] In some embodiments, the anti-CD19 CAR comprises (i) an ectodomain that comprises an anti-CD19 scFv comprising variable heavy and light chain regions comprising the amino acid sequences set forth in SEQ ID NOs: 152 and 153, respectively, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a CD28 co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 20 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0343] In some embodiments, the anti-CD19 CAR comprises the amino acid sequence set forth in SEQ ID NO: 149. In some embodiments, the anti-CD19 CAR is encoded by the nucleotide sequence set forth in SEQ ID NO: 148. In some embodiments, the anti-CD19 CAR is encoded by a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to the nucleotide sequence set forth in SEQ ID NO: 148.

CD33 CAR

[0344] In some embodiments, the engineered T cells described herein comprise a CD33 targeting CAR, also referred to herein as CD33 CAR, anti-CD33 CAR or anti-CD33 CAR T cells. In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 antigen-binding domain, (ii) a transmembrane domain, and (iii) an endodomain comprising at least one co-stimulatory domain.

[0345] In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 or 41BB co-stimulatory domain, and a CD3-zeta signaling domain. In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 co-stimulatory domain and a CD3-zeta signaling domain. In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a 41BB co-stimulatory domain and a CD3-zeta signaling domain.

[0346] In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 antigen-binding domain, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0347] In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 scFv comprising the amino acid sequence set forth in SEQ ID NO: 137, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0348] In some embodiments, the anti-CD33 CAR comprises (i) an ectodomain that comprises an anti-CD33 scFv comprising variable heavy and light chain regions comprising the amino acid sequences set forth in SEQ ID NOs: 140 and 141, respectively, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0349] In some embodiments, the anti-CD33 CAR comprises the amino acid sequence set forth in SEQ ID NO: 139. In some embodiments, the anti-CD33 CAR is encoded by the nucleotide sequence set forth in SEQ ID NO: 136. In some embodiments, the anti-CD33 CAR is encoded by a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to the nucleotide sequence set forth in SEQ ID NO: 136.

BCMA CAR

[0350] In some embodiments, the engineered T cells described herein comprise a BCMA targeting CAR, also referred to herein as BCMA CAR, anti-BCMA CAR or anti-BCMA CAR T cells. In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA antigen-binding domain, (ii) a transmembrane domain, and (iii) an endodomain comprising at least one co-stimulatory domain.

[0351] In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 or 41BB co-stimulatory domain, and a CD3-zeta signaling domain. In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 co-stimulatory domain and a CD3-zeta signaling domain. In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a 41BB co-stimulatory domain and a CD3-zeta signaling domain.

[0352] In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA antigen-binding domain, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0353] In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA scFv comprising the amino acid sequence set forth in SEQ ID NO: 59, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0354] In some embodiments, the anti-BCMA CAR comprises (i) an ectodomain that comprises an anti-BCMA scFv comprising variable heavy and light chain regions comprising the amino acid sequences set forth in SEQ ID NOs: 60 and 61, respectively, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0355] In some embodiments, the anti-BCMA CAR comprises the amino acid sequence set forth in SEQ ID NO: 57. In some embodiments, the anti-BCMA CAR is encoded by the nucleotide sequence set forth in SEQ ID NO: 56. In some embodiments, the anti-BCMA CAR is encoded by a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to the nucleotide sequence set forth in SEQ ID NO: 56.

CD70 CAR

[0356] In some embodiments, the engineered T cells described herein comprise a CD70 targeting CAR, also referred to herein as CD70 CAR, anti-CD70 CAR or anti-CD70 CAR T cells. In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 antigen-binding domain, (ii) a transmembrane domain, and (iii) an endodomain comprising at least one co-stimulatory domain.

[0357] In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 or 41BB co-stimulatory domain, and a CD3-zeta signaling domain. In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a CD28 co-stimulatory domain and a CD3-zeta signaling domain. In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 antigen-binding domain, (ii) a CD8 transmembrane domain, and (iii) an endodomain that comprises a 41BB co-stimulatory domain and a CD3-zeta signaling domain.

[0358] In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 antigen-binding domain, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0359] In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 scFv comprising the amino acid sequence set forth in SEQ ID NO: 50, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0360] In some embodiments, the anti-CD70 CAR comprises (i) an ectodomain that comprises an anti-CD70 scFv comprising variable heavy and light chain regions comprising the amino acid sequences set forth in SEQ ID NOs: 51 and 52, respectively, (ii) a CD8 transmembrane domain comprising the amino acid sequence set forth in SEQ ID NO: 126, and (iii) an endodomain that comprises a 41BB co-stimulatory domain comprising the amino acid sequence set forth in SEQ ID NO: 19 and a CD3-zeta signaling domain comprising the amino acid sequence set forth in SEQ ID NO: 22.

[0361] In some embodiments, the anti-CD70 CAR comprises the amino acid sequence set forth in SEQ ID NO: 46. In some embodiments, the anti-CD70 CAR is encoded by the nucleotide sequence set forth in SEQ ID NO: 45. In some embodiments, the anti-CD70 CAR is encoded by a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to the nucleotide sequence set forth in SEQ ID NO: 45.

Expression of Chimeric Antigen Receptor Construct

Donor Template

[0362] The nucleic acid encoding a CAR may be delivered to a T cell that comprises what is referred to herein as a donor template (also referred to as a donor polynucleotide). A donor template can contain a non-homologous sequence, such as the nucleic acid encoding a CAR, flanked by two regions of homology to allow for efficient HDR at a genomic location of interest. Alternatively, a donor template may have no regions of homology to the targeted location in the DNA and may be integrated by NHEJ-dependent end joining following cleavage at the target site.

[0363] A donor template can be DNA or RNA, single-stranded and/or double-stranded, and can be introduced into a cell in linear or circular form. If introduced in linear form, the ends of the donor sequence can be protected (e.g., from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are added to the 3′ terminus

of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends. See, for example, Chang et al., (1987) Proc. Natl. Acad. Sci. USA 84:4959-4963; Nehls et al., (1996) Science 272:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues.

[0364] A donor template can be introduced into a cell as part of a vector molecule having additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance. Moreover, a donor template can be introduced as naked nucleic acid, as nucleic acid complexed with an agent such as a liposome or poloxamer, or can be delivered by viruses (e.g., adenovirus, AAV, herpesvirus, retrovirus, lentivirus and integrase defective lentivirus (IDLV)).

[0365] A donor template, in some embodiments, is inserted so that its expression is driven by the endogenous promoter at the integration site, namely the promoter that drives expression of the endogenous gene into which the donor is inserted. However, in some embodiments, the donor template comprises an exogenous promoter and/or enhancer, for example a constitutive promoter, an inducible promoter, or tissue-specific promoter. In some embodiments, the exogenous promoter is an EF1α promoter comprising a sequence of SEQ ID NO: 123. Other promoters may be used.

[0366] Furthermore, exogenous sequences may also include transcriptional or translational regulatory sequences, for example, promoters, enhancers, insulators, internal ribosome entry sites, sequences encoding 2A peptides and/or polyadenylation signals.

[0367] In some embodiments, the donor template comprises a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 98% identity to SEQ ID NO: 44. In some embodiments, the donor template comprises the nucleotide sequence of SEQ ID NO: 44.

[0368] In some embodiments, the donor template comprises a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 98% identity to SEQ ID NO: 55. In some embodiments, the donor template comprises the nucleotide sequence of SEQ ID NO: 55.

[0369] In some embodiments, the donor template comprises a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 98% identity to SEQ ID NO: 135. In some embodiments, the donor template comprises the nucleotide sequence of SEQ ID NO: 135.

[0370] In some embodiments, the donor template comprises a nucleotide sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 98% identity to SEQ ID NO: 156. In some embodiments, the donor template comprises the nucleotide sequence of SEQ ID NO: 156.

Other Methods

[0371] In some embodiments, a nucleic acid encoding a CAR is introduced into an engineered cell by methods known to those of skill in the art. For example, a CAR may be introduced into an engineered cell by a vector. A variety of different methods known in the art can be used to introduce any of the nucleic acids or expression vectors disclosed herein into an immune effector cell. Non-limiting examples of methods for introducing nucleic acid into a cell include: lipofection, transfection (e.g., calcium phosphate transfection, transfection using highly branched organic compounds, transfection using cationic polymers, dendrimer-based transfection, optical transfection, particle-based transfection (e.g., nanoparticle transfection), or transfection using liposomes (e.g., cationic liposomes)), microinjection, electroporation, cell squeezing, sonoporation, protoplast fusion, impalefection, hydrodynamic delivery, gene gun, magnetofection, viral transfection, and nucleofection.

Delivery Methods and Constructs

[0372] Nucleases and/or donor templates may be delivered using a vector system, including, but not limited to, plasmid vectors, DNA minicircles, retroviral vectors, lentiviral vectors, adenovirus vectors, poxvirus vectors; herpesvirus vectors and adeno-associated virus vectors, and combinations thereof.

[0373] Conventional viral and non-viral based gene transfer methods can be used to introduce

nucleic acids encoding nucleases and donor templates in cells (e.g., T cells). Non-viral vector delivery systems include DNA plasmids, DNA minicircles, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell.

[0374] Methods of non-viral delivery of nucleic acids include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, naked RNA, capped RNA, artificial virions, and agent-enhanced uptake of DNA. Sonoporation using, e.g., the Sonitron 2000 system (Rich-Mar) can also be used for delivery of nucleic acids. Some specific examples are provided below.

Adeno-Associated Viral Delivery

[0375] The donor nucleic acid encoding a CAR construct can be delivered to a cell using an adeno-associated virus (AAV). AAVs are small viruses which integrate site-specifically into the host genome and can therefore deliver a transgene, such as CAR. Inverted terminal repeats (ITRs) are present flanking the AAV genome and/or the transgene of interest and serve as origins of replication. Also present in the AAV genome are rep and cap proteins which, when transcribed, form capsids which encapsulate the AAV genome for delivery into target cells. Surface receptors on these capsids which confer AAV serotype, which determines which target organs the capsids will primarily bind and thus what cells the AAV will most efficiently infect. There are twelve currently known human AAV serotypes. In some embodiments, the AAV is AAV serotype 6 (AAV6).

[0376] Adeno-associated viruses are among the most frequently used viruses for gene therapy for several reasons. First, AAVs do not provoke an immune response upon administration to mammals, including humans. Second, AAVs are effectively delivered to target cells, particularly when consideration is given to selecting the appropriate AAV serotype. Finally, AAVs have the ability to infect both dividing and non-dividing cells because the genome can persist in the host cell without integration. This trait makes them an ideal candidate for gene therapy.

Homology-Directed Repair (HDR)

[0377] The donor nucleic acid encoding a CAR is inserted by homology directed repair (HDR) into the target gene locus. Both strands of the DNA at the target locus are cut by a CRISPR Cas9 enzyme. HDR then occurs to repair the double-strand break (DSB) and insert the donor DNA. For this to occur correctly, the donor sequence is designed with flanking residues which are complementary to the sequence surrounding the DSB site in the target gene (hereinafter "homology arms"). These homology arms serve as the template for DSB repair and allow HDR to be an essentially error-free mechanism. The rate of homology directed repair (HDR) is a function of the distance between the mutation and the cut site so choosing overlapping or nearby target sites is important. Templates can include extra sequences flanked by the homologous regions or can contain a sequence that differs from the genomic sequence, thus allowing sequence editing.

[0378] The target gene can be associated with an immune response in a subject, wherein permanently deleting at least a portion of the target gene will modulate the immune response. For example, to generate a CAR T cell, the target gene can be the TCRα constant region (TRAC). Disruption of TRAC leads to loss of function of the endogenous TCR.

[0379] In some embodiments, the target gene is in a safe harbor locus.

Engineered T Cells

[0380] Engineered (gene edited) CAR T cells of the present disclosure may be autologous ("self") or non-autologous ("non-self," e.g., allogeneic, syngeneic or xenogeneic). "Autologous" refers to cells from the same subject. "Allogeneic" refers to cells of the same species as a subject, but that differ genetically to the cells in the subject. In some embodiments, the T cells are obtained from a mammal. In some embodiments, the T cells are obtained from a human.

[0381] 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.

[0382] In some embodiments, an isolated population of T cells is used. In some embodiments, after isolation of peripheral blood mononuclear cells (PBMC), both cytotoxic and helper T lymphocytes can be sorted into naive, memory, and effector T cell subpopulations either before or after activation, expansion, and/or genetic modification.

[0383] A specific subpopulation of T cells, expressing one or more of the following cell surface markers: TCRab, CD3, CD4, CD8, CD27 CD28, CD38 CD45RA, CD45RO, CD62L, CD127, CD122, CD95, CD197, CCR7, KLRG1, MCH-I proteins and/or MCH-II proteins, can be further isolated by positive or negative selection techniques. In some embodiments, a specific subpopulation of T cells, expressing one or more of the markers selected from the group consisting of TCRab, CD4 and/or CD8, is further isolated by positive or negative selection techniques. In some embodiments, the engineered T cell populations do not express or do not substantially express one or more of the following markers: CD70, CD57, CD244, CD160, PD-1, CTLA4, HM3, and LAG3. In some embodiments, subpopulations of T cells may be isolated by positive or negative selection prior to genetic engineering and/or post genetic engineering.

[0384] In some embodiments, an isolated population of T cells expresses one or more of the markers including, but not limited to a CD3+, CD4+, CD8+, or a combination thereof. In some embodiments, the T cells are isolated from a donor, or subject, and first activated and stimulated to proliferate in vitro prior to undergoing gene editing.

[0385] 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. Pat. Nos. 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. In some 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 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.

[0386] In some embodiments, T cells are activated and expanded for about 4 hours, about 6 hours, about 12 hours, about 18 hours, about 24 hours, about 36 hours, about 48 hours, about 60 hours, or about 72 hours prior to introduction of the gene editing compositions into the T cells.

[0387] In some embodiments, T cells are activated at the same time that genome editing compositions are introduced into the T cells. T cell populations or isolated T cells generated by any of the gene editing methods described herein are also within the scope of the present disclosure.

[0388] In some embodiments, provided herein is a population of T cells comprising genetically engineered T cells, which comprise a disrupted endogenous CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR), e.g., those described herein. In some embodiments, the CAR binds an antigen expressed on a pathological cell. In some embodiments, the CAR binds CD70. In other embodiments, the CAR does not bind CD70. Such a T cell population may further comprise genetically engineered T cells having one or more of the following gene edits: a disrupted endogenous programmed cell death-1 (PD-1) gene, a disrupted endogenous T cell receptor alpha chain constant region (TRAC) gene, and a disrupted endogenous beta-2-microglobulin (β2M) gene. In some examples, the nucleic acid encoding the CAR may be inserted into the TRAC locus.

[0389] In some embodiments, the population of T cells disclosed herein comprises genetically engineered T cells, which comprise a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR) that binds an antigen expressed on a pathological cell. In some embodiments, the population of T cells disclosed herein comprises genetically engineered T cells, which comprise a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor

(CAR), wherein the CAR binds CD70. In other embodiments, the population of T cells disclosed herein comprises genetically engineered T cells that comprise a disrupted CD70 gene and a nucleic acid encoding a CAR, wherein the CAR does not bind CD70. In some embodiments, the population of T cells disclosed herein comprises genetically engineered T cells, which comprise a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR) that binds an antigen expressed on a pathological cell, and further comprises a disrupted PD1 gene. In some embodiments, the CAR binds CD70. In some embodiments the CAR does not bind CD70. In some aspects, the CAR binds CD19. In some embodiments, the CAR binds CD33. In some aspects, the CAR binds BCMA. Any of the just-noted engineered T cells may further comprise a disrupted T cell receptor alpha chain constant region (TRAC) gene and/or a disrupted beta-2-microglobulin (β2M) gene.

[0390] In particular examples, provided herein is a population of T cells comprising genetically engineered T cells, which comprise a disrupted CD70 gene, a disrupted T cell receptor alpha chain constant region (TRAC) gene, a disrupted beta-2-microglobulin (β2M) gene, a nucleic acid encoding a chimeric antigen receptor (CAR), e.g., an anti-BCMA CAR, anti-CD19 CAR, anti-CD33 CAR, or anti-CD70 CAR as described herein, and optionally a disrupted programmed cell death-1 (PD-1) gene. Any of the engineered T cells disclosed herein may contain native (undisrupted) HLA genes.

[0391] In some examples, at least 50% (e.g., 60%, 70%, 80%, 90%, or 95%) of the population of T cells express the CAR as disclosed herein and do not express a detectable level of surface CD70. Such cells may further possess the features of not expressing a detectable level of surface TCR, a detectable level of surface β2M, and/or a detectable level of surface PD-1. For example, at least 50% (e.g., 60%, 70%, 80%, 90%, or 95%) of the population of T cells express the CAR as disclosed herein and do not express a detectable level of surface CD70, a detectable level of surface TCR, and a detectable level of surface β2M. In some instances, at least 50% (e.g., 60%, 70%, 80%, 90%, or 95%) of the population of T cells express the CAR as disclosed herein and do not express a detectable level of surface CD70, a detectable level of surface TCR, a detectable level of surface β2M, and a detectable level of PD-1.

[0392] An isolated cell expressing the CAR as described herein and does not express a detectable level of surface CD70 is also within the scope of the present disclosure. Such an isolated cell may not express a detectable level of surface TCR, a detectable level of surface β2M, and/or a detectable level of surface PD-1. In some examples, the isolated cell comprises a nucleic acid encoding the CAR, which is inserted into the TRAC locus.

[0393] Also provided herein are an engineered T cell population comprising engineered T cells comprising an RNA-guided nuclease, e.g., those described herein (for example, a Cas9 nuclease), and a guide RNA (gRNA) targeting a CD70 gene (e.g., those described herein). In some instances, at least 50% (e.g., 60%, 70%, 80%, 90%, or 95%) of the T cells in the T cell population comprise the RNA-guided nuclease and the gRNA targeting the CD70 gene. Such an engineered T cell population may further comprise engineered T cells comprising a gRNA targeting a PD-1 gene, a gRNA targeting a TRAC gene, a gRNA targeting a β2M gene, and/or a nucleic acid (e.g., a vector) comprising a donor template that comprises a nucleotide sequence encoding a CAR (e.g., those described herein), which optionally is flanked by left and right homology arms to the TRAC gene locus. In some examples, at least 50% (e.g., 60%, 70%, 80%, 90%, or 95%) of the T cells in the T cell population comprise the RNA-guided nuclease, the gRNA targeting the CD70 gene, and the nucleic acid coding for the CAR. When the nucleic acid coding for the CAR further comprises the left and right homology arms to the TRAC gene locus, the T cells may also comprise a gRNA targeting the TRAC gene. In addition, the T cells may further comprise a gRNA targeting a PD-1 gene, a gRNA targeting a β2M gene, or a combination thereof.

[0394] Also within the scope of the present disclosure is an isolated engineered T cell comprising the RNA-guided nuclease, the gRNA targeting the CD70 gene, and optionally one or more of a

gRNA targeting a PD-1 gene, a gRNA targeting a TRAC gene, a gRNA targeting a β2M gene, and a nucleic acid (e.g., a vector) comprising a donor template that comprises a nucleotide sequence encoding a CAR (e.g., those described herein). The nucleotide sequence encoding the CAR may be flanked by left and right homology arms to the TRAC gene locus.

Generating CAR-T Cells

[0395] In some embodiments, the engineered T cells described herein are generated by modifying the genome of the cells. In some embodiments, a double stranded break (DSB) at a site in a target gene is induced. In some embodiments, the DSB is repaired using one or more endogenous DNA repair pathways. In some embodiments, a DNA repair pathway does not require a homologous sequence (e.g., the non-homologous end joining pathway or NHEJ pathway). In some embodiments, a repair pathway requires a homologous sequence (e.g., the homology-directed pathway or HDR pathway).

[0396] In some embodiments, the engineered T cells described herein are generated by inducing a DSB with CRISPR-Cas9 as an endonuclease, and one or more non-coding RNAs, and repairing the DSB using HDR and a donor polynucleotide template described herein.

[0397] In some embodiments, the engineered T cells described herein are generated using a gRNA complimentary to a sequence of a target gene that is a TRAC. In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA spacer comprising the sequence set forth in SEQ ID NO: 98. In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA comprising the sequence set forth in SEQ ID NO: 30. In some embodiments, the TRAC gRNA comprising the sequence set forth in SEQ ID NO: 98 targets the TRAC sequence set forth in SEQ ID NO: 118. In some embodiments, the TRAC gRNA comprising the sequence set forth in SEQ ID NO: 30 targets the TRAC sequence set forth in SEQ ID NO: 118.

[0398] In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA spacer comprising the sequence set forth in SEQ ID NO: 108. In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA comprising the sequence set forth in SEQ ID PO: 40. In some embodiments, the TRAC gRNA comprising the sequence set forth in SEQ ID NO: 108 targets the TRAC sequence set forth in SEQ ID NO: 118. In some embodiments, the TRAC gRNA comprising the sequence set forth in SEQ ID NO: 40 targets the TRAC sequence set forth in SEQ ID NO: 118.

[0399] In some embodiments, the engineered T cells described herein are generated using a gRNA complimentary to a sequence of a target gene that is a β2M. In some embodiments, the engineered T cells described herein are generated using a β2M gRNA spacer comprising the sequence set forth in SEQ ID NO: 99. In some embodiments, the engineered T cells described herein are generated using a β2M gRNA comprising the sequence set forth in SEQ ID NO: 31. In some embodiments, the β2M gRNA comprising the sequence set forth in SEQ ID NO: 99 targets the β2M sequence set forth in SEQ ID NO: 119. In some embodiments, the β2M gRNA comprising the sequence set forth in SEQ ID NO: 31 targets the β2M sequence set forth in SEQ ID NO: 119.

[0400] In some embodiments, the engineered T cells described herein are generated using a β2M gRNA spacer comprising the sequence set forth in SEQ ID NO: 109. In some embodiments, the engineered T cells described herein are generated using a β2M gRNA comprising the sequence set forth in SEQ ID NO: 41. In some embodiments, the β2M gRNA comprising the sequence set forth in SEQ ID NO: 109 targets the β2M sequence set forth in SEQ ID NO: 119. In some embodiments, the β2M gRNA comprising the sequence set forth in SEQ ID NO: 41 targets the β2M sequence set forth in SEQ ID NO: 119.

[0401] In some embodiments, the engineered T cells described herein are generated using a gRNA complimentary to a sequence of a target gene that is a CD70. In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA spacer comprising the sequence set forth in SEQ ID NO: 94. In some embodiments, the engineered T cells described herein are

generated using a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 26. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 94 targets the CD70 sequence set forth in SEQ ID NO: 114. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 26 targets the CD70 sequence set forth in SEQ ID NO: 114.

[0402] In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA spacer comprising the sequence set forth in SEQ ID NO: 104. In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 36. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 104 targets the CD70 sequence set forth in SEQ ID NO: 114. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 36 targets the CD70 sequence set forth in SEQ ID NO: 114.

[0403] In some embodiments, the engineered T cells described herein are generated using a gRNA complimentary to a sequence of a target gene that is a CD70. In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA spacer comprising the sequence set forth in SEQ ID NO: 95. In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 27. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 95 targets the CD70 sequence set forth in SEQ ID NO: 115. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 27 targets the CD70 sequence set forth in SEQ ID NO: 115.

[0404] In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA spacer comprising the sequence set forth in SEQ ID NO: 105. In some embodiments, the engineered T cells described herein are generated using a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 37. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 105 targets the CD70 sequence set forth in SEQ ID NO: 115. In some embodiments, the CD70 gRNA comprising the sequence set forth in SEQ ID NO: 37 targets the CD70 sequence set forth in SEQ ID NO: 115.

[0405] In some embodiments, the engineered T cells described herein are generated using a gRNA complimentary to a sequence of a target gene that is a PD-1. In some embodiments, the engineered T cells described herein are generated using a PD-1 gRNA spacer comprising the sequence set forth in SEQ ID NO: 100. In some embodiments, the engineered T cells described herein are generated using a PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 32. In some embodiments, the PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 100 targets the β2M sequence set forth in SEQ ID NO: 120. In some embodiments, the PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 32 targets the PD-1 sequence set forth in SEQ ID NO: 120.

[0406] In some embodiments, the engineered T cells described herein are generated using a PD-1 gRNA spacer comprising the sequence set forth in SEQ ID NO: 110. In some embodiments, the engineered T cells described herein are generated using a PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 42. In some embodiments, the PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 110 targets the PD-1 sequence set forth in SEQ ID NO: 120. In some embodiments, the PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 42 targets the PD-1 sequence set forth in SEQ ID NO: 120.

[0407] In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA comprising the sequence set forth in SEQ ID NO: 98, a β2M gRNA comprising the sequence set forth in SEQ ID NO: 99, a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 94 or 95, and/or a PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 100. In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA comprising the sequence set forth in SEQ ID NO: 108, a β2M gRNA comprising the sequence set forth in SEQ ID NO: 109, a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 104 or 105, and/or a PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 110.

[0408] In some embodiments, the engineered T cells described herein are generated using a TRAC

gRNA comprising the sequence set forth in SEQ ID NO: 30, a β2M gRNA comprising the sequence set forth in SEQ ID NO: 31, a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 26 or 27, and/or a PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 32. In some embodiments, the engineered T cells described herein are generated using a TRAC gRNA comprising the sequence set forth in SEQ ID NO: 40, a β2M gRNA comprising the sequence set forth in SEQ ID NO: 41, a CD70 gRNA comprising the sequence set forth in SEQ ID NO: 36 or 27, and/or a PD-1 gRNA comprising the sequence set forth in SEQ ID NO: 42.

[0409] In some embodiments, the engineered T cells are generated using a donor template comprising a non-homologous sequence that is a nucleic acid encoding a CAR. In some embodiments, a donor template is comprised of homology arms that correspond to sequences in a target gene that is a TRAC. In some embodiments, a 5′ homology arm (left homology arm) of the donor template comprises the sequence set forth in SEQ ID NO: 122. In some embodiments, a 3′ homology arm of the donor template comprises the sequence set forth in SEQ ID NO: 125.

[0410] In some embodiments, an exogenous promoter is an EF1a promoter comprises the sequence set forth in SEQ ID NO: 123. In some embodiments, a donor template comprises the sequence set forth in SEQ ID NO: 135. In some embodiments, a donor template comprises the sequence set forth in SEQ ID NO: 156. In some embodiments, a donor template comprises the sequence set forth in SEQ ID NO: 44. In some embodiments, a donor template comprises the sequence set forth in SEQ ID NO: 55.

[0411] In some embodiments, polynucleotides encoding gRNAs, nucleases, and donor templates are introduced into cells (e.g., T cells) using conventional viral and non-viral based gene transfer methods.

[0412] In some embodiments, a polynucleotide such as a gRNA, a sgRNA, an mRNA encoding a nuclease, or a donor template are delivered to a cell using a non-viral vector delivery system. Examples of a non-viral vector delivery system include, but are not limited to, a DNA plasmid, a DNA minicircle, a naked nucleic acid, a liposome, a ribonucleoprotein particle (RNP) or a poloxamer. In some embodiments, a method of introducing polynucleotides to a cell using a non-viral vector delivery system includes electroporation, lipofection, microinjection, biolistics, or agent-enhanced uptake.

[0413] In some embodiments, a polynucleotide such as a gRNA, a sgRNA, an mRNA encoding a nuclease, or a donor template are delivered to a cell using a viral vector delivery system. Examples of a viral vector delivery system include, but are not limited to, retroviral vectors, lentiviral vectors, adenovirus vectors, poxvirus vectors, herpesvirus vectors, and adeno-associated virus (AAV) vectors.

[0414] In some embodiments, a donor template encoding a CAR construct is delivered to a cell as one or more polynucleotides. In some embodiments, a donor template encoding a CAR construct is delivered by a viral delivery vehicle. In some embodiments, a viral delivery vehicle is an adeno-associated virus (AAV) vector.

[0415] In some embodiments, an endonuclease (e.g., Cas9) is delivered to a cell as a polypeptide. In some embodiments, an endonuclease (e.g., Cas9) is delivered to a cell separately from a genome-targeting nucleic acid (e.g., a gRNA, a sgRNA). In some embodiments, an endonuclease (e.g., Cas9) is delivered to a cell as a complex with one or more genome-targeting polynucleotides (e.g., a gRNA, a sgRNA). In some embodiments, a endonuclease or a pre-complexed endonuclease is delivered by a non-viral delivery vehicle that includes, but is not limited to, a nanoparticle, a liposome, a ribonucleoprotein, a positively charged peptide, a small molecule RNA-conjugate, an aptamer-RNA chimeras, or an RNA-fusion protein complex. In some embodiments, a method of introducing an endonuclease polypeptide or a pre-complexed endonuclease polypeptide to a cell includes electroporation, lipofection, microinjection, biolistics, or agent-enhanced uptake.

[0416] In some embodiments, a Cas9 polypeptide is pre-complexed with one or more sgRNAs to form a ribonucleoprotein particle (RNP). In some embodiments, a Cas9/sgRNA RNP is formulated

using a lipid nanoparticle. In some embodiments, a donor template is formulated using an AAV vector. In some embodiments, delivery to a cell of a formulated Cas9/sgRNA RNP is performed by electroporation of the cell. In some embodiments, a donor template formulated as an AAV vector is delivered prior to electroporation. In some embodiments, a donor template formulated as an AAV vector is delivered during electroporation. In some embodiments, a donor template formulated as an AAV vector is delivered following electroporation.

[0417] In some embodiments, a gene edit performed using a CRISPR/Cas9 endonuclease results in an engineered T cell with a disrupted TRAC gene. In some embodiments, a disruption of a TRAC gene results in eliminated or decreased expression of the TRAC gene product. In some embodiments, a disruption of a TRAC gene disrupts or inhibits transcription and translation of an encoded gene product. In some embodiments, a disruption of a TRAC gene results in eliminated or decreased expression of a TRAC gene product. In some embodiments, eliminated or decreased expression of the TRAC gene is associated with loss of function of the TCR. In some embodiments, loss of TCR function renders an engineered T cell suitable to allogeneic transplantation (i.e., minimizing the risk of inducing GvHD). In some embodiments, a disruption of a TRAC gene is created by knocking in a CAR into the TRAC gene (e.g., using an AAV vector and a donor template). In some embodiments, a disruption in the TRAC gene expression is created by gRNAs targeting the TRAC genomic region and knocking in a CAR into the CAR gene. In some embodiments, a knock-in CAR is provided by a donor template with homology arms that correspond to sequences of the TRAC surrounding the site of a DSB.

[0418] In some embodiments, a gene edit performed using a CRISPR/Cas9 endonuclease results in an engineered T cell with a disrupted β2M gene. In some embodiments, gRNAs targeting the B2M genomic region create indels in the β2M gene that disrupt or inhibit transcription and translation of an encoded gene product. In some embodiments, a disruption of a β2M gene results in eliminated or decreased expression of the β2M polypeptide. In some embodiments, eliminated or decreased expression of the B2M polypeptide is associated with loss of function of the MHC I complex. In some embodiments, loss of MHC I function renders an engineered T cell suitable to allogeneic transplantation (i.e., minimizing the risk of a host versus allogeneic T cell response). In some embodiments, loss of MHC I function results in increased persistence of an engineered T cell in an allogeneic recipient.

[0419] In some embodiments, a gene edit performed using a CRISPR/Cas9 endonuclease results in an engineered T cell with a disrupted CD70 gene. In some embodiments, gRNAs targeting the CD70 genomic region create indels in the CD70 gene that disrupt or inhibit transcription and translation of an encoded gene product. In some embodiments, a disruption of a CD70 gene results in eliminated or decreased expression of the CD70 polypeptide. In some embodiments, eliminated or decreased expression of the CD70 polypeptide is associated with enhanced cell proliferation, enhanced in vivo persistence, decreased exhaustion, and/or enhanced anti-tumor efficacy.

[0420] In some embodiments, a gene edit performed using a CRISPR/Cas9 endonuclease results in an engineered T cell with a disrupted PD-1 gene. In some embodiments, gRNAs targeting the PD-1 genomic region create indels in the PD-1 gene that disrupt or inhibit transcription and translation of an encoded gene product. In some embodiments, a disruption of a PD-1 gene results in eliminated or decreased expression of the PD-1 polypeptide.

Methods and Compositions

[0421] Provided herein, in some embodiments, are methods for treating cancer. Non-limiting examples of cancers that may be treated as provided herein include multiple myeloma, leukemia (e.g., T cell leukemia, B-cell acute lymphoblastic leukemia (B-ALL), and/or chronic lymphocytic leukemia (C-CLL)), lymphoma (e.g., B-cell non-Hodgkin's lymphoma (B-NHL), Hodgkin's lymphoma, and/or T cell lymphoma), and/or clear cell renal cell carcinoma (ccRCC). In some embodiment, the methods comprise delivering the CAR T cells (e.g., anti-BCMA, anti-CD19, anti-CD33 and/or anti-CD70 CAR T cells) of the present disclosure to a subject having multiple

myeloma, leukemia, or lymphoma. Other non-limiting examples of cancers (e.g., solid tumors) that may be treated as provided herein include pancreatic cancer, gastric cancer, ovarian cancer, cervical cancer, breast cancer, renal cancer, thyroid cancer, nasopharyngeal cancer, non-small cell lung (NSCLC), glioblastoma, and/or melanoma.

[0422] CD70 has also been detected on hematological tumors and on carcinomas. The restricted expression pattern of CD70 in normal tissues and its widespread expression in various malignancies makes it an attractive target for antibody-based therapeutics. The use of CAR T cell therapy to target CD70.sup.+ cancers, however, is potentially problematic because of CD70 expression in the T cells. To address this potential problem, the present disclosure also provides CAR T cells that have been engineered to disrupt endogenous CD70 expression while at the same time expressing an anti-CD70 binding moiety (e.g., an anti-CD70 scFv).

[0423] In some embodiments, the cancer is a CD70.sup.+ cancer. In other embodiments, the cancer is a BCMA.sup.+ cancer. In some embodiments, the cancer is a CD19+ cancer. In some embodiments, the cancer is a CD33+ cancer. It should be understood that other cancers, expressing other cancer antigens, may be treated using the engineered CD70 knockout CAR T cells of the present disclosure.

[0424] The methods, in some embodiments, comprise administering to a subject (e.g., a patient having a CD70.sup.+ cancer, a BCMA.sup.+ cancer, a CD19+ cancer or a CD33+ cancer) a population of CAR T cells as provided herein. In some embodiments, the methods comprise administering to a subject a population of CAR T cells comprising a CD70 gene knockout. In some embodiments, the methods comprise administering to a subject a population of CAR T cells comprising a CD70 gene knockout and a PD1 gene knockout. In some embodiments, the methods comprise implanting the cells into subject. This implanting step may be accomplished using any method of implantation known in the art. For example, the engineered cells may be injected directly in a subject's blood or otherwise administered to the subject.

[0425] As demonstrated herein, CAR T cells comprising a CD70 gene knockout exhibit extended proliferation and increased in vivo persistence. In some embodiments, CAR T cells comprising a CD70 gene knockout exhibit increased anti-tumor efficacy relative to CAR T cells expressing endogenous CD70. In some embodiments, CAR T cells comprising a CD70 gene knockout exhibit increased anti-tumor efficacy in solid tumors relative to CAR T cells expressing endogenous CD70. Without wishing to be bound by theory, the increased in vivo persistence of CAR T cells comprising a CD70 gene knockout may allow for expansion in solid tumors and therefore provide enhanced anti-tumor efficacy in such tumors relative to CAR T cells expressing endogenous CD70.

[0426] In some embodiments, the disclosure provides a method for treating a solid tumor with the CAR T cells described herein. In some embodiments, the disclosure provides a method for treating a solid tumor with the anti-CD70 CAR T cells described herein.

[0427] The step of administering may include the placement (e.g., transplantation) of cells, e.g., engineered T cells, into a subject, by a method or route that results in at least partial localization of the introduced cells at a desired site, such as tumor, such that a desired effect(s) is produced. Engineered T cells can be administered by any appropriate route that results in delivery to a desired location in the subject where at least a portion of the implanted cells or components of the cells remain viable. The period of viability of the cells after administration to a subject can be as short as a few hours, e.g., twenty-four hours, to a few days, to as long as several years, or even the life time of the subject, i.e., long-term engraftment. For example, in some aspects described herein, an effective amount of engineered T cells is administered via a systemic route of administration, such as an intraperitoneal or intravenous route.

[0428] A subject may be any subject for whom diagnosis, treatment, or therapy is desired. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human.

[0429] A donor is an individual who is not the subject being treated. A donor is an individual who is not the patient. In some embodiments, a donor is an individual who does not have or is not

suspected of having the cancer being treated. In some embodiments, multiple donors, e.g., two or more donors, are used.

[0430] In some embodiments, an engineered T cell population being administered according to the methods described herein comprises allogeneic T cells obtained from one or more donors. Allogeneic refers to a cell, cell population, or biological samples comprising cells, obtained from one or more different donors of the same species, where the genes at one or more loci are not identical to the recipient (e.g., subject). For example, an engineered T cell population, being administered to a subject can be derived from one or more unrelated donors, or from one or more non-identical siblings. In some embodiments, syngeneic cell populations may be used, such as those obtained from genetically identical donors, (e.g., identical twins). In some embodiments, the cells are autologous cells; that is, the engineered T cells are obtained or isolated from a subject and administered to the same subject, i.e., the donor and recipient are the same.

[0431] An effective amount refers to the amount of a population of engineered T cells needed to prevent or alleviate at least one or more signs or symptoms of a medical condition (e.g., cancer), and relates to a sufficient amount of a composition to provide the desired effect, e.g., to treat a subject having a medical condition. An effective amount also includes an amount sufficient to prevent or delay the development of a symptom of the disease, alter the course of a symptom of the disease (for example but not limited to, slow the progression of a symptom of the disease), or reverse a symptom of the disease. It is understood that for any given case, an appropriate effective amount can be determined by one of ordinary skill in the art using routine experimentation.

[0432] For use in the various aspects described herein, an effective amount of cells (e.g., engineered T cells) comprises at least 10^2 cells, at least $5×10^2$ cells, at least 10^3 cells, at least $5×10^3$ cells, at least 10^4 cells, at least $5×10^4$ cells, at least 10^5 cells, at least $2×10^5$ cells, at least $3×10^5$ cells, at least $4×10^5$ cells, at least $5×10^5$ cells, at least $6×10^5$ cells, at least $7×10^5$ cells, at least $8×10^5$ cells, at least $9×10^5$ cells, at least $1×10^6$ cells, at least $2×10^6$ cells, at least $3×10^6$ cells, at least $4×10^6$ cells, at least $5×10^6$ cells, at least $6×10^6$ cells, at least $7×10^6$ cells, at least $8×10^6$ cells, at least $9×10^6$ cells, or multiples thereof. The cells are derived from one or more donors, or are obtained from an autologous source. In some examples described herein, the cells are expanded in culture prior to administration to a subject in need thereof.

[0433] Modes of administration include injection, infusion, instillation, or ingestion. Injection includes, without limitation, intravenous, intramuscular, intra-arterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. In some embodiments, the route is intravenous.

[0434] In some embodiments, engineered T cells are administered systemically, which refers to the administration of a population of cells other than directly into a target site, tissue, or organ, such that it enters, instead, the subject's circulatory system and, thus, is subject to metabolism and other like processes.

[0435] The efficacy of a treatment comprising a composition for the treatment of a medical condition can be determined by the skilled clinician. A treatment is considered "effective treatment," if any one or all of the signs or symptoms of, as but one example, levels of functional target are altered in a beneficial manner (e.g., increased by at least 10%), or other clinically accepted symptoms or markers of disease (e.g., cancer) are improved or ameliorated. Efficacy can also be measured by failure of a subject to worsen as assessed by hospitalization or need for medical interventions (e.g., progression of the disease is halted or at least slowed). Methods of measuring these indicators are known to those of skill in the art and/or described herein. Treatment includes any treatment of a disease in subject and includes: (1) inhibiting the disease, e.g., arresting, or slowing the progression of symptoms; or (2) relieving the disease, e.g., causing regression of symptoms; and (3) preventing or reducing the likelihood of the development of

symptoms.

[0436] Combination therapies are also encompassed by the present disclosure. For example, CD70 and/or CD27 antibodies can be used to bind and/or modulate the activity of CD70 and/or CD27 on CAR T cells and promote a decrease in exhaustion, enhanced CAR T cell expansion and increase efficacy of cancer cell killing. Thus, CD70 and/or CD27 antibodies can be administered with any CAR T cell known in the art to improve the CAR T cell function. For example, any of the engineered T cells provided herein may be administered in combination with anti-CD70 antibodies, anti-CD27 antibodies, or a combination of anti-CD70 antibodies and anti-CD27 antibodies. In some embodiments, TRAC.sup.−/β2M.sup.− CAR.sup.+ T cells (e.g., anti-CD70 CAR or anti-BCMA CAR) are administered in combination with anti-CD70 and/or anti-CD27 antibodies. In some embodiments, TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.− CAR.sup.+ T cells (e.g., anti-CD70 CAR or anti-BCMA CAR) are administered in combination with anti-CD70 and/or anti-CD27 antibodies. In some embodiments, TRAC.sup.−/β2M.sup.−/PD-1.sup.− CAR.sup.+ T cells (e.g., anti-CD70 CAR or anti-BCMA CAR) are administered in combination with anti-CD70 and/or anti-CD27 antibodies. In some embodiments, TRAC.sup.−/β2M.sup.−/CD70.sup.− CAR.sup.+ T cells (e.g., anti-CD70 CAR or anti-BCMA CAR) are administered in combination with anti-CD70 and/or anti-CD27 antibodies. In some embodiments, the antibodies administered in combination can be Varlilumab.

[0437] In some embodiments, the disclosure provides a method of reducing exhaustion of T cells comprising disrupting the CD70 gene in the T cells. In some embodiments, the disclosure provides a method of increasing proliferation of T cells comprising disrupting the CD70 gene in the T cells. In some embodiments, the disclosure provides a method of increasing cytotoxicity of T cells comprising disrupting the CD70 gene in the T cells. In some embodiments, the disclosure provides a method of overcoming inhibitory effect of an immune checkpoint (e.g., PD-1) in T cells comprising disrupting the CD70 gene in the T cells.

OTHER EMBODIMENTS

[0438] The disclosure relates to the following embodiments. Throughout this section, the term embodiment is abbreviated as 'E' followed by an ordinal. For example, E1 is equivalent to Embodiment 1.

[0439] E1. An engineered T cell comprising a disrupted CD70 gene, a disrupted programmed cell death-1 (PD-1) gene, and a nucleic acid encoding a chimeric antigen receptor (CAR).

[0440] E2. An engineered T cell comprising a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR) that binds CD70.

[0441] E3. An engineered T cell comprising a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR) that does not bind CD70.

[0442] E4. The engineered T cell of embodiment 2 or 3, further comprising a disrupted PD-1 gene.

[0443] E5. The engineered T cell of any one of embodiments 1-4 further comprising a disrupted T cell receptor alpha chain constant region (TRAC) gene.

[0444] E6. The engineered T cell of any one of embodiments 1-5 further comprising a disrupted beta-2-microglobulin (β2M) gene.

[0445] E7. An engineered T cell, comprising [0446] a disrupted T cell receptor alpha chain constant region (TRAC) gene; [0447] a disrupted beta-2-microglobulin (β2M) gene; [0448] a disrupted CD70 gene; and [0449] a nucleic acid encoding a chimeric antigen receptor (CAR).

[0450] E8. The engineered T cell of embodiment 7, wherein the nucleic acid encoding the CAR is inserted into the TRAC gene.

[0451] E9. The engineered T cell of embodiment 7 or 8, further comprising a disrupted PD-1 gene.

[0452] E10. The engineered T cell of any one of embodiments 1-9, wherein the CAR comprises an ectodomain that comprises an anti-CD70 antibody, optionally wherein the anti-CD70 antibody is an anti-CD70 single-chain variable fragment (scFv).

[0453] E11. The engineered T cell of embodiment 10, wherein the anti-CD70 scFv comprises the

same heavy chain variable region (VH) complementarity determining regions (CDRs) and the same light chain variable region (VL) CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 51 and a VL set forth as SEQ ID NO: 52.

[0454] E12. The engineered T cell of embodiment 11, wherein the anti-CD70 scFv comprises the same VH and VL chains as the reference antibody.

[0455] E13. The engineered T cell of embodiment 11, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 48 or 50.

[0456] E14. The engineered T cell of embodiment 11, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 50.

[0457] E15. The engineered T cell of any one of embodiments 1-9, wherein the CAR comprises an ectodomain that comprises an anti-BCMA antibody, optionally wherein the anti-BCMA antibody is an anti-BCMA single-chain variable fragment (scFv).

[0458] E16. The engineered T cell of embodiment 15, wherein the anti-BCMA scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH) set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61.

[0459] E17. The engineered T cell of embodiment 16, wherein the anti-BCMA scFv comprises the same VH and VL chains as the reference antibody.

[0460] E18. The engineered T cell of embodiment 16, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59.

[0461] E19. The engineered T cell of any one of embodiments 1-18, wherein the CAR comprises a CD28 or 41BB co-stimulatory domain and optionally a CD3ζ signaling domain.

[0462] E20. The engineered T cell of any one of embodiments 5-19, wherein the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 44 or 55 and/or the nucleic acid encoding the CAR comprises the nucleotide sequence of SEQ ID NO: 45 or 56.

[0463] E21. The engineered T cell of any one of embodiments 6-20, wherein the disrupted β2M gene comprises gene at least one nucleotide sequence selected from any one of SEQ ID NOS: 9-14.

[0464] E22. The engineered T cell of any one of embodiments 1-21, wherein the engineered T cell maintains cytotoxicity following 5 rechallenges with a cancer cell.

[0465] E23. The engineered T cell of embodiment 22, wherein the engineered T cell maintains cytotoxicity following 10 rechallenges with a cancer cell.

[0466] E24. A population of cells comprising engineered T cells that comprise a disrupted CD70 gene, a disrupted programmed cell death-1 (PD-1) gene, and a nucleic acid encoding a chimeric antigen receptor (CAR).

[0467] E25. A population of cells comprising engineered T cells that comprise a disrupted CD70 gene, and a nucleic acid encoding a chimeric antigen receptor (CAR) that binds CD70.

[0468] E26. A population of cells comprising engineered T cells that comprise a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR) that does not bind CD70.

[0469] E27. The population of cells of embodiment 25 or 26 further comprising a disrupted programmed cell death-1 (PD-1) gene.

[0470] E28. The population of cells of any one of embodiments 24-27 further comprising a disrupted T cell receptor alpha chain constant region (TRAC) gene.

[0471] E29. The population of cells of any one of embodiments 24-28 further comprising a disrupted beta-2-microglobulin (β2M) gene.

[0472] E30. A population of cells comprising [0473] engineered T cells that comprise [0474] a disrupted T cell receptor alpha chain constant region (TRAC) gene; [0475] a disrupted beta-2-microglobulin (β2M) gene; [0476] a disrupted CD70 gene; and [0477] a nucleic acid encoding a chimeric antigen receptor (CAR).

[0478] E31. The population of cells of embodiment 30, wherein the nucleic acid encoding the CAR is inserted into the TRAC gene.

[0479] E32. The population of cells of embodiment 30 or 31, wherein the engineered T cells further comprise a disrupted programmed cell death-1 (PD-1) gene.

[0480] E33. The population of cells of any one of embodiments 24 or 27-32, wherein the CAR comprises an ectodomain that comprises an anti-CD70 antibody, optionally wherein the anti-CD70 antibody is an anti-CD70 single-chain variable fragment (scFv).

[0481] E34. The population of cells of embodiment 33, wherein the anti-CD70 scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 51 and a VL set forth as SEQ ID NO: 52.

[0482] E35. The population of cells of embodiment 34, wherein the anti-CD70 scFv comprises the same VH and VL chains as the reference antibody.

[0483] E36. The population of cells of embodiment 35, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 48 or 50.

[0484] E37. The population of cells of embodiment 35, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 50.

[0485] E38. The population of cells of any one of embodiments 24-32, wherein the CAR comprises an ectodomain that comprises an anti-BCMA antibody, optionally wherein the anti-BCMA antibody is an anti-BCMA single-chain variable fragment (scFv).

[0486] E39. The population of cells of embodiment 38, wherein the anti-BCMA scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61.

[0487] E40. The population of cells of embodiment 39, wherein the anti-BCMA scFv comprises the same VH and VL chains as the reference antibody.

[0488] E41. The population of cells of embodiment 39, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59.

[0489] E42. The population of cells of any one of embodiments 28-41, wherein the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 44 or 55 and/or the nucleic acid encoding the CAR comprises the nucleotide sequence of SEQ ID NO: 45 or 56.

[0490] E43. The population of cells of any one of embodiments 29-42, wherein the disrupted β2M gene comprises gene at least one nucleotide sequence selected from any one of SEQ ID NOS: 9-14.

[0491] E44. The population of cells of any one of embodiments 24-43, wherein at least 50% of the engineered T cells do not express a detectable level of TCR surface protein, do not express a detectable level of β2M surface protein, do not express a detectable level of CD70 surface protein, do not express a detectable level of PD-1 surface protein, and/or express a detectable level of the CAR.

[0492] E45. The population of cells of embodiment 44, wherein 50%-70%, of the engineered T cells do not express a detectable level of TCR surface protein, do not express a detectable level of β2M surface protein, do not express a detectable level of CD70 surface protein, do not express a detectable level of PD-1 surface protein, and/or express a detectable level of the CAR.

[0493] E46. The population of cells of any one of embodiments 28-45, wherein at least 90%, optionally 90%-100%, of the engineered T cells do not express a detectable level of TCR surface protein.

[0494] E47. The population of cells of any one of embodiments 29-46, wherein at least 60%, optionally 60%-75%, of the engineered T cells do not express a detectable level of β2M surface protein.

[0495] E48. The population of cells of any one of embodiments 24-47, wherein at least 80%, optionally 80%-100%, of the engineered T cells do not express a detectable level of CD70 surface protein.

[0496] E49. The population of cells of any one of embodiments 1-48, wherein at least 80%,

optionally 80%-95%, of the engineered T cells express a detectable level of the CAR.

[0497] E50. The population of cells of any one of embodiments 24-49, wherein the engineered T cells exhibit at least 20% greater cellular proliferative capacity, relative to control T cells.

[0498] E51. The population of cells of any one of embodiments 24-50, wherein the engineered T cells exhibit at least 20% greater cellular lysis capability, relative to control T cells.

[0499] E52. The population of cells of any one of embodiments 24-51, wherein the level of cytokines secreted by the engineered T cells are at least 2-fold greater than the level of cytokines secreted by control T cells.

[0500] E53. The population of any one of embodiments 24-52, wherein the engineered T cells exhibit reduced cellular exhaustion, relative to control T cells.

[0501] E54. The population of cells of embodiment 53, wherein the engineered T cells express reduced levels of LAG3, relative to control T cells.

[0502] E55. The population of cells of any one of embodiments 54, wherein the control T cells are engineered T cells that express endogenous CD70 protein.

[0503] E56. The population of cells of any one of embodiments 24-55, wherein the engineered T cells maintain cytokine-dependent proliferation.

[0504] E57. The population of cells of any one of embodiments 24-56, wherein the engineered T cells maintain cytotoxicity following 5 rechallenges with a cancer cell.

[0505] E58. The population of cells of embodiment 47, wherein the engineered T cells maintain cytotoxicity following 10 rechallenges with a cancer cell.

[0506] E59. A method comprising administering to a subject the population of cells of any one of embodiments 24-58.

[0507] E60. The method of embodiment 59, wherein the engineered T cells are engineered human T cells.

[0508] E61. The method of embodiment 59 or 60, wherein the subject has a cancer.

[0509] E62. The method of embodiment 61, wherein the cancer expresses CD70 and/or BCMA.

[0510] E63. The method of any one of embodiments 59-62, wherein the population of cells is administered to the subject in an amount effective to treat the cancer.

[0511] E64. The method of any one of embodiments 59-63, wherein the cancer is a solid tumor malignancy or a hematological malignancy.

[0512] E65. The method embodiment 64, wherein the solid tumor malignancy is selected from the group consisting of: ovarian tumor, pancreatic tumor, kidney tumor, lung tumor, and intestinal tumor.

[0513] E66. The method of embodiment 63, wherein the population of cells is administered to the subject in an amount effective to reduce the volume of a tumor in the subject.

[0514] E67. A method for producing an engineered T cell, the method comprising [0515] (a) delivering to a T cell [0516] an RNA-guided nuclease, [0517] a gRNA targeting a CD70 gene, and [0518] a vector comprising a donor template that comprises a nucleic acid encoding a CAR; and [0519] (b) producing an engineered T cell comprising a disrupted CD70 gene and expressing the CAR.

[0520] E68. The method of embodiment 67, further comprising in step (a) delivering to the T cell a gRNA targeting a PD-1 gene; wherein the engineered T cell of step (b) further comprises a disrupted PD-1 gene.

[0521] E69. The method of embodiment 67 or embodiment 68, further comprising in step (a) delivering to the T cell a gRNA targeting a TRAC gene; wherein the engineered T cell of step (b) further comprises a disrupted TRAC gene.

[0522] E70. The method of embodiment 69, wherein the nucleic acid encoding the CAR is flanked by left and right homology arms to the TRAC gene locus; and wherein the engineered T cell of step (b) comprises the nucleic acid encoding the CAR inserted into the TRAC gene locus.

[0523] E71. The method of any one of embodiments embodiment 67-70, further comprising in step

(a) delivering to the T cell a gRNA targeting a β2M gene; wherein the engineered T cell of step (b) further comprises a disrupted β2M gene.

[0524] E72. A method for producing an engineered T cell, the method comprising [0525] (a) delivering to a T cell [0526] an RNA-guided nuclease, [0527] a gRNA targeting a TRAC gene, [0528] a gRNA targeting a β2M gene, [0529] a gRNA targeting a CD70 gene, and [0530] a vector comprising a donor template that comprises a nucleic acid encoding a CAR; and [0531] (b) producing an engineered T cell.

[0532] E73. The method of embodiment 72, wherein the nucleic acid encoding the CAR is flanked by left and right homology arms to the TRAC gene locus.

[0533] E74. The method of embodiment 72 or 73 further comprising delivering to the T cell a gRNA targeting a PD-1 gene.

[0534] E75. The method of any one of embodiments 67-74, wherein the RNA-guided nuclease is a Cas9 nuclease, optionally a *S. pyogenes* Cas9 nuclease.

[0535] E76. The method of any one of embodiments 69-75, wherein the gRNA targeting the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 98 or targets the nucleotide sequence of SEQ ID NO: 118, and optionally wherein the gRNA targeting the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 30.

[0536] E77. The method of any one of embodiments 71-76, wherein the gRNA targeting the β2M gene comprises the nucleotide sequence of SEQ ID NO: 99 or targets the nucleotide sequence of SEQ ID NO: 119, and optionally wherein the gRNA targeting the β2M gene comprises the nucleotide sequence of SEQ ID NO: 31.

[0537] E78. The method of any one of embodiments 67-77, wherein the gRNA targeting the CD70 gene comprises the nucleotide sequence of SEQ ID NOS: 94 or 95 or targets the nucleotide sequence of SEQ ID NO: 114 or 115, and optionally wherein the gRNA targeting the CD70 gene comprises the nucleotide sequence of SEQ ID NOS: 26 or 27.

[0538] E79. The method of any one of embodiments 68-71 and 74-78, wherein the gRNA targeting the PD-1 gene comprises the nucleotide sequence of SEQ ID NO: 100 or targets the nucleotide sequence of SEQ ID NO: 120, and optionally wherein the gRNA targeting the PD-1 gene comprises the nucleotide sequence of SEQ ID NO: 32.

[0539] E80. The method of any one of embodiments 67-79, wherein the CAR comprises an ectodomain that comprises an anti-CD70 antibody, optionally wherein the anti-CD70 antibody is an anti-CD70 single-chain variable fragment (scFv).

[0540] E81. The method of embodiment 80, wherein the anti-CD70 scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 51 and a VL set forth as SEQ ID NO: 52.

[0541] E82. The method of embodiment 81, wherein the anti-CD70 scFv comprises the same VH and VL chains as the reference antibody.

[0542] E83. The method of embodiment 81, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 48 or 50.

[0543] E84. The method of embodiment 81, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 50.

[0544] E85. The method of any one of embodiments 67-79, wherein the CAR comprises an ectodomain that comprises an anti-BCMA antibody, optionally wherein the anti-BCMA antibody is an anti-BCMA single-chain variable fragment (scFv).

[0545] E86. The method of embodiment 85, wherein the anti-BCMA scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61.

[0546] E87. The method of embodiment 86, wherein the anti-BCMA scFv comprises the same VH

and VL chains as the reference antibody.

[0547] E88. The method of embodiment 86, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59.

[0548] E89. The method of any one of embodiments 67-88, wherein the CAR further comprises a CD28 or 41BB co-stimulatory domain and optionally a CD3z signaling domain.

[0549] E90. The method of embodiment 72, wherein the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 46.

[0550] E91. The method of embodiment 72, wherein the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 57.

[0551] E92. An engineered T cell comprising an RNA-guided nuclease and a gRNA targeting a CD70 gene, optionally wherein the gRNA targeting the CD70 gene comprises the nucleotide sequence of SEQ ID NOS: 94 or 95 or targets the nucleotide sequence of SEQ ID NO: 114 or 115, and optionally wherein the gRNA targeting the CD70 gene comprises the nucleotide sequence of SEQ ID NOS: 26 or 27.

[0552] E93. The engineered T cell of embodiment 92 further comprising a gRNA targeting a PD-1 gene, optionally wherein the gRNA targeting the PD-1 gene comprises the nucleotide sequence of SEQ ID NO: 100 or targets the nucleotide sequence of SEQ ID NO: 120, and optionally wherein the gRNA targeting the PD-1 gene comprises the nucleotide sequence of SEQ ID NO: 32.

[0553] E94. The engineered T cell of embodiment 92 or 93 further comprising a gRNA targeting a TRAC gene, optionally wherein the gRNA targeting the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 98 or targets the nucleotide sequence of SEQ ID NO: 118, and optionally wherein the gRNA targeting the TRAC gene comprises the nucleotide sequence of SEQ ID NO: 30.

[0554] E95. The engineered T cell of any one of embodiments 92-94 further comprising a gRNA targeting a β2M gene, optionally wherein the gRNA targeting the 82M gene comprises the nucleotide sequence of SEQ ID NO: 99 or targets the nucleotide sequence of SEQ ID NO: 119, and optionally wherein the gRNA targeting the β2M gene comprises the nucleotide sequence of SEQ ID NO: 31.

[0555] E96. The engineered T cell of any one of embodiments 92-95, wherein the RNA-guided nuclease is a Cas9 nuclease, optionally a *S. pyogenes* Cas9 nuclease.

[0556] E97. The engineered T cell of any one of embodiments 92-96 further comprising a vector comprising a donor template that comprises a nucleic acid encoding a CAR, optionally wherein the nucleic acid encoding the CAR is flanked by left and right homology arms to the TRAC gene locus.

[0557] E98. The engineered T cell of embodiment 97, wherein the CAR comprises an ectodomain that comprises an anti-CD70 antibody, optionally wherein the anti-CD70 antibody is an anti-CD70 single-chain variable fragment (scFv).

[0558] E99. The engineered T cell of embodiment 98, wherein the anti-CD70 scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 51 and a VL set forth as SEQ ID NO: 52.

[0559] E100. The engineered T cell of embodiment 99, wherein the anti-CD70 scFv comprises the same VH and VL chains as the reference antibody.

[0560] E101. The engineered T cell of embodiment 99, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 48 or 50.

[0561] E102. The engineered T cell of embodiment 99, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 50.

[0562] E103. The engineered T cell of embodiment 97 wherein the CAR comprises an ectodomain that comprises an anti-BCMA antibody, optionally wherein the anti-BCMA antibody is an anti-BCMA single-chain variable fragment (scFv).

[0563] E104. The engineered T cell of embodiment 103, wherein the anti-BCMA scFv comprises

the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61.

[0564] E105. The engineered T cell of embodiment 104, wherein the anti-BCMA scFv comprises the same VH and VL chains as the reference antibody.

[0565] E106. The engineered T cell of embodiment 104, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59.

[0566] E107. The engineered T cell of embodiment 97, wherein the vector comprises a nucleic acid encoding a CAR that comprises the amino acid sequence of SEQ ID NO: 46 or 57.

[0567] E108. A method of increasing proliferation or reducing exhaustion of T cells, the method comprising disrupting the CD70 gene in the T cells.

[0568] E109. The method of embodiment 108 further comprising disrupting in the T cells at least one gene selected from the group consisting of: programmed cell death-1 (PD-1) gene, T cell receptor alpha chain constant region (TRAC) gene, and beta-2-microglobulin (β2M) gene.

[0569] E110. The method of any one of embodiments 108-109 further comprising expressing in the T cells a nucleic acid encoding a chimeric antigen receptor (CAR).

[0570] E111. The method of any one of embodiments 108-110, wherein the CD70 gene is disrupted by CRISPR/Cas gene editing.

[0571] E112. The method of any one of embodiments 110-111, wherein the PD-1, TRAC, and/or β2M gene is disrupted by CRISPR/Cas gene editing.

[0572] E113. A method for treating cancer in a subject, comprising administering to the patient a population of cells comprising engineered T cells, wherein the engineered T cells comprise a disrupted CD70 gene and a nucleic acid encoding a CAR, thereby treating cancer in the subject.

[0573] E114. The method of embodiment 113, wherein the CAR binds CD70.

[0574] E115. The method of embodiment 113, wherein the CAR does not bind CD70.

[0575] E116. The method of any one of embodiments 113-115, wherein the engineered T cells further comprise a disrupted TRAC gene.

[0576] E117. The method of any one of embodiments 113-116, wherein the engineered T cells further comprise a disrupted B2M gene.

[0577] E118. The method of any one of embodiments 113-116, wherein the engineered T cells further comprise a disrupted PD-1 gene.

[0578] E119. A method for treating cancer in a subject, comprising administering to the patient a population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0579] (i) a disrupted TRAC gene; [0580] (ii) a disrupted B2M gene; [0581] (iii) a disrupted CD70 gene; and [0582] (iv) a nucleic acid encoding a CAR; [0583] thereby treating the cancer in the subject.

[0584] E120. The method of any one of embodiments 113-114 and 116-119, wherein the CAR comprises (a) an ectodomain that comprises an anti-CD70 antigen-binding fragment, (b) a CD8 transmembrane domain, and (c) an endodomain that comprises a 41BB co-stimulatory domain and a CD3z co-stimulatory domain.

[0585] E121. The method of embodiment 119 or 120, wherein the disrupted TRAC gene comprises the nucleic acid encoding the CAR.

[0586] E122. The method of any one of embodiments 120-121, wherein the anti-CD70 antibody is an anti-CD70 scFv.

[0587] E123. The method of embodiment 122, wherein the anti-CD70 scFv comprises the same heavy chain variable region (VH) complementarity determining regions (CDRs) and the same light chain variable region (VL) CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 51 and a VL set forth as SEQ ID NO: 52.

[0588] E124. The method of embodiment 123, wherein the anti-CD70 scFv comprises the same VH and VL chains as the reference antibody.

[0589] E125. The method of embodiment 122, wherein the anti-CD70 scFv comprises the amino

acid sequence of SEQ ID NO: 48 or 50.

[0590] E126. The method of embodiment 122, wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 50.

[0591] E127. The method of any one of embodiments 113 and 115-118, wherein the CAR comprises an ectodomain that comprises an anti-BCMA antibody, optionally wherein the anti-BCMA antibody is an anti-BCMA single-chain variable fragment (scFv).

[0592] E128. The method of embodiment 127, wherein the anti-BCMA scFv comprises the same VH complementarity determining regions (CDRs) and the same VL CDRs as a reference antibody, wherein the reference antibody comprises a VH) set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61.

[0593] E129. The method of embodiment 128, wherein the anti-BCMA scFv comprises the same VH and VL chains as the reference antibody.

[0594] E130. The method of embodiment 127, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59.

[0595] E131. The method of any one of embodiments 113-130, wherein the engineered T cells are engineered human T cells.

[0596] E132. The method of any one of embodiments 113-131, wherein the cancer expresses CD70 and/or BCMA.

[0597] E133. The method of any one of embodiments 113-132, wherein the population of cells is administered to the subject in an amount effective to treat the cancer.

[0598] E134. The method of any one of embodiments 113-133, wherein the cancer is a solid tumor malignancy or a hematological malignancy.

[0599] E135. The method embodiment 134, wherein the solid tumor malignancy is selected from the group consisting of: ovarian tumor, pancreatic tumor, kidney tumor, lung tumor, and intestinal tumor.

[0600] E136. A population of cells comprising engineered T cells, wherein the engineered T cells comprise: [0601] (i) a disrupted TRAC gene; [0602] (ii) a disrupted beta-2-microglobulin (B2M) gene; [0603] (iii) a disrupted CD70 gene [0604] (iv) a nucleic acid encoding a CAR comprising (a) an ectodomain that comprises an anti-CD70 antigen-binding fragment, (b) a CD8 transmembrane domain, and (c) an endodomain that comprises a 41BB co-stimulatory domain and a CD3z co-stimulatory domain.

[0605] E137. The population of cells of embodiment 136, wherein the disrupted TRAC gene comprises the nucleic acid encoding the CAR.

[0606] E138. The population of cells of any one of embodiments 136-137, wherein the engineered T cells are human T cells.

[0607] E139. An engineered T cell comprising: [0608] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR comprising the amino acid sequence set forth in SEQ ID NO: 46; [0609] (ii) a disrupted B2M gene; and [0610] (iii) a disrupted CD70 gene.

[0611] E140. The engineered T cell of embodiment 139, wherein the nucleic acid encoding the CAR comprises a sequence at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 45.

[0612] E141. An engineered T cell comprising: [0613] (i) a disrupted TRAC gene, wherein the disrupted TRAC gene comprises a nucleic acid encoding a CAR, wherein the nucleic acid sequence is at least 90% identical to SEQ ID NO: 45; [0614] (ii) a disrupted B2M gene; and [0615] (iii) a disrupted CD70 gene.

[0616] E142. The engineered T cell of any one of embodiments 139-141, wherein the disrupted TRAC gene comprises a donor sequence comprising the nucleotide sequence set forth in SEQ ID NO: 45 or SEQ ID NO: 44.

[0617] E143. An engineered T cell comprising: [0618] (i) a disrupted TRAC gene comprising the

nucleic acid sequence of SEQ ID NO: 44; [0619] (ii) a disrupted B2M gene; and [0620] (iii) a disrupted CD70 gene.

[0621] E144. The engineered T cell of any one of embodiments 139-143, wherein the T cell is a human T cell.

EXAMPLES

Example 1. Efficient Knockout of CD70 by Cas9:sgRNA RNPs in T Cells

[0622] This example describes efficient editing of the CD70 gene in primary human T cells ex vivo using CRISPR/Cas9 gene editing. Genomic segments of the CD70 gene containing the first three (3) protein coding exons were used as input in gRNA design software. The genomic segments also included flanking splice site acceptor/donor sequences. Desired gRNAs were those that would lead to insertions or deletions in the coding sequence, disrupting the amino acid sequence of CD70, leading to out of frame/loss of function allele(s) (referred to as "CD70 knockout" alleles). All seven (7) in silico-identified gRNA spacer sequences targeting the CD70 gene were synthesized, and the gRNAs were specifically modified, as indicated in Table 5. While the gRNAs in Table 5 were modified with 2′-O-methyl phosphorothioate modifications, unmodified gRNAs, or gRNAs with other modifications, may be used. See also PCT/IB2018/001619, filed May 11, 2018, herein incorporated in its entirety by this reference.

TABLE-US-00005 TABLE 5 CD70 gRNA Sequences/Target Sequences gRNA Sequences Name Unmodified Sequence Modified Sequence CD70 sgRNA (E1_T1) UCACCAAGCCCGCGACCAAUg U*C*A*CCAAGCCCGCGACCA uuuuagagcuagaaauagcaaguuaaaaua AUguuuuagagcuagaaauagcaaguuaa aggcuaguccguuaucaacuugaaaaagug aauaaggcuaguccguuaucaacuugaaaa gcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 23) (SEQ ID NO: 33) CD70 sgRNA (E1_T1) spacer UCACCAAGCCCGCGACCAAU U*C*A*CCAAGCCCGCGACCA (SEQ ID NO: 91) AU (SEQ ID NO: 101) CD70 sgRNA (E1_T3) AUCACCAAGCCCGCGACCAAg A*U*C*ACCAAGCCCGCGACC uuuuagagcuagaaauagcaaguuaaaaua AAguuuuagagcuagaaauagcaaguuaa aggcuaguccguuaucaacuugaaaaagug aauaaggcuaguccguuaucaacuugaaaa gcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 24) (SEQ ID NO: 34) CD70 sgRNA (E1_T3) spacer AUCACCAAGCCCGCGACCAA A*U*C*ACCAAGCCCGCGACC (SEQ ID NO: 92) AA (SEQ ID NO: 102) CD70 sgRNA (E1_T4) CGGUGCGGCGCAGGCCCUAU C*G*G*UGCGGCGCAGGCCCU guuuuagagcuagaaauagcaaguuaaaau AUguuuuagagcuagaaauagcaaguuaa aaggcuaguccguuaucaacuugaaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 25) (SEQ ID NO: 35) CD70 sgRNA (E1_T4) spacer CGGUGCGGCGCAGGCCCUAU C*G*G*UGCGGCGCAGGCCCU (SEQ ID NO: 93) AU (SEQ ID NO: 103) CD70 sgRNA (E1_T7)); also GCUUUGGUCCCAUUGGUCGC G*C*U*UUGGUCCCAUUGGUC referred to as: T7 guuuuagagcuagaaauagcaaguuaaaau GCguuuuagagcuagaaauagcaaguuaa aaggcuaguccguuaucaacuugaaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 26) (SEQ ID NO: 36) CD70 sgRNA (E1_T7) spacer GCUUUGGUCCCAUUGGUCGC G*C*U*UUGGUCCCAUUGGUC (SEQ ID NO: 94) GC (SEQ ID NO: 104) CD70 sgRNA (E1_T8); also GCCCGCAGGACGCACCCAUAg G*C*C*CGCAGGACGCACCCA referred to as: T8 uuuuagagcuagaaauagcaaguuaaaaua UAguuuuagagcuagaaauagcaaguuaa aggcuaguccguuaucaacuugaaaaagug aauaaggcuaguccguuaucaacuugaaaa gcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 27) (SEQ ID NO: 37) CD70 sgRNA (E1_T8) spacer GCCCGCAGGACGCACCCAUA G*C*C*CGCAGGACGCACCCA (SEQ ID NO: 95) UA (SEQ ID NO: 105) CD70 sgRNA (E1_T10) GUGCAUCCAGCGCUUCGCAC G*U*G*CAUCCAGCGCUUCGC

guuuuagagcuagaaauagcaaguuaaaau ACguuuuagagcuagaaauagcaaguuaa
aaggcuaguccguuaucaacuugaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU
aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 28) (SEQ ID NO: 38) CD70
sgRNA (E1_T10) spacer GUGCAUCCAGCGCUUCGCA C
G*U*G*CAUCCAGCGCUUCGC (SEQ ID NO: 96) AC (SEQ ID NO: 106) CD70
sgRNA (E3_T1) CAGCUACGUAUCCAUCGUGA C*A*G*CUACGUAUCCAUCGU
guuuuagagcuagaaauagcaaguuaaaau GAguuuuagagcuagaaauagcaaguuaa
aaggcuaguccguuaucaacuugaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU
aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 29) (SEQ ID NO: 39) CD70
sgRNA (E3_T1) spacer CAGCUACGUAUCCAUCGUGA C*A*G*CUACGUAUCCAUCGU
(SEQ ID NO: 97) GA (SEQ ID NO: 107) Target Sequences Name Target
Sequence (PAM) CD70 sgRNA (E1_T1) TCACCAAGCCCGCGACCAAT (GGG)
(SEQ ID NO: 111) CD70 sgRNA (E1_T3) ATCACCAAGCCCGCGACCAA (TGG)
(SEQ ID NO: 112) CD70 sgRNA (E1_T4) CGGTGCGGCGCAGGCCCTAT (GGG)
(SEQ ID NO: 113) CD70 sgRNA (E1_T7) GCTTTGGTCCCATTGGTCGC (GGG)
(SEQ ID NO: 114) CD70 sgRNA (E1_T8) GCCCGCAGGACGCACCCATA (GGG)
(SEQ ID NO: 115) CD70 sgRNA (E1_T10) GTGCATCCAGCGCTTCGCAC (AGG)
(SEQ ID NO: 116) CD70 sgRNA (E3_T1) CAGCTACGTATCCATCGTGA (TGG)
(SEQ ID NO: 117) TRAC sgRNA AGAGCAACAGTGCTGTGGCC (TGG) (SEQ
ID NO: 118) β2M sgRNA GCTACTCTCTCTTTCTGGCC (TGG) (SEQ ID NO:
119) PD-1 sgRNA CTGCAGCTTCTCCAACACAT (CGG) (SEQ ID NO: 120) *2′-O-
methyl phosphorothioate residue

[0623] Primary human T cells were transfected (electroporated) with a ribonucleoprotein particle (RNP) containing Cas9 nuclease and a synthetic modified sgRNA targeting the CD70 gene (sequences in Table 5) or controls (no Cas9, no gRNA). Four to six (4-6) days post transfection, cells were (1) subjected to a TIDE analysis to assess indel frequency and (2) processed by flow cytometry (primary antibody: FITC anti-human CD70 antibody, clone 113-16, Biolegend) to assess CD70 expression levels at the cell surface.

[0624] Seven (7) gRNAs yielded measurable data by TIDE analysis, as indicated in Table 6. Four (4) gRNA sequences yielded indel percentages (editing frequencies) above 85% with protein expression knockdown above 80% (SEQ ID NOS: 23, 26, 27 and 29), indicating highly efficient gene editing. The data in Table 6 are from one (1) donor. The level of CD70 protein expression (assessed by median fluorescent intensity (MFI)) per test sample was normalized to the level of CD70 protein expression present in control cells.

TABLE-US-00006 TABLE 6 CD70 gRNA sequences, cutting efficiencies, and CD70 surface protein expression in gene edited T cells Protein expres- sion knock- down gRNA Name gRNA Spacer Sequence Indel % R.sup.2 % CD70 UCACCAAGCCCGCGACCAAU 89.3% 0.97 84.8% EXON1_T1 (SEQ ID NO: 91) (E1_T1) CD70 AUCACCAAGCCCGCGACCAA 65.2% 0.93 84.0% EXON1_T3 (SEQ ID NO: 92) (E1_T3) CD70 CGGUGCGGCGCAGGCCCUAU 81.6% 0.83 87.5% EXON1_T4 (SEQ ID NO: 93) (E1_T4) CD70 GCUUUGGUCCCAUUGGUCGC 97.8% 0.98 87.7% EXON1_T7 (SEQ ID NO: 94) (E1_T7) CD70 GCCCGCAGGACGCACCCAUA 90.1% 0.94 88.1% EXON1_T8 (SEQ ID NO: 95) (E1_T8) CD70 GUGCAUCCAGCGCUUCGCAC 28.3% 0.30 83.9% EXON1_T10 (SEQ ID NO: 96) (E1_T10) CD70 CAGCUACGUAUCCAUCGUGA 85.6% 0.93 87.2% EXON3_T1 (SEQ ID NO: 97) (E3_T1)

Analysis of On-Target Indel Profiles in T Cells

[0625] On-target amplicon analysis was conducted at the CD70 locus following gene editing using the T7 guide (SEQ ID NO: 26; SEQ ID NO: 36), targeting the CD70 gene: GCTTTGGTCCCATTGGTCGC (SEQ ID NO: 160; target sequence, with PAM SEQ ID NO: 114).

[0626] Following gene editing, on-target amplicon analysis was conducted around the CD70 locus in TRAC−/β2M−/CD70−/anti-CD70 CAR+ cells (generated as described in Example 3).

[0627] An initial PCR was performed using the KAPA HiFi PCR kit (Kapa Biosystems, Wilmington, MA). 100 ng of input gDNA was combined with 10 uM of each primer. The CD70_F and CD70_R primers were paired to amplify the CD70 locus (Table 7).

TABLE-US-00007 TABLE 7 Primers for CD70 amplicon library preparation CD70_F TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGcccaacttt tccatctcaactcaccccaagtg (SEQ ID NO: 127) CD70_R GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGcccctcct gcgctagcgga (SEQ ID NO: 128)

[0628] Analysis of the CD70 locus in a population of T cells following CRISPR/Cas9 gene editing to produce TRAC.sup.−/β2M.sup.−/anti-CD70 CAR+ T cells results in specific indel frequencies and edited gene sequences at the CD70 locus (Table 8; deletions as dashes and insertions in bold). Two cell populations of edited cells were generated from two different donor T cells (1 and 2). The populations of edited T cells from each donor were analyzed in replicate: 1A/1B and 2A/2B.

TABLE-US-00008 TABLE 8

SEQ ID Std. NO:	Gene Edited Sequence	1A	1B	2A	2B	Mean	Dev.
129	CACACCACGAGGCAGATCACCAAGCCCGCG-- CAATGGGACCAAAGCAGCCCGCAGGACG	10.4%	11.1%	14.4%	14.8%	12.7%	0.022
130	CACACCACGAGGCAGATCACCAAGCCCGCG**A** ACCAATGGGACCAAAGCAGCCCGCAGGACG	8.7%	10.0%	11.3%	11.1%	10.3%	0.012
131	CACACCACGAGGCAGATC------------ ACCAATGGGACCAAAGCAGCCCGCAGGACG	8.2%	7.8%	7.1%	6.8%	7.5%	0.006
132	CACACCACGAGGCAGATCACCAAGCCCGCG- CCAATGGGACCAAAGCAGCCCGCAGGACG	3.9%	4.5%	4.2%	4.3%	4.2%	0.002
133	CACACCACGAGGCAGATCACCAAGCCCGC- ACCAATGGGACCAAAGCAGCCCGCAGGACG	2.2%	2.5%	2.4%	2.6%	2.4%	0.002
134	CACACCACGAGGCAGATCACCA------------------ ------AGCCCGCAGGACG	2.9%	2.3%	2.0%	2.0%	2.3%	0.004

Example 2. Generation of T Cells with Multiple Gene Knockouts

[0629] This example describes the use of CRISPR/Cas9 gene editing technology to produce human T cells that lack expression of two, three or four genes simultaneously. Specifically, the T cell receptor (TCR) gene (gene edited in the TCR Alpha Constant (TRAC) region), the β2-microglobulin (β2M) gene, the Cluster of Differentiation 70 (CD70) gene and/or the programmed cell death 1 (PD-1 or PD1) gene were edited by CRISPR/Cas9 gene editing to produce T cells deficient in two or more of the listed genes. The following abbreviations are used in the Figures for brevity and clarity: [0630] 2×KO: TRAC.sup.−/β2M.sup.− [0631] 3×KO (PD-1): TRAC.sup.−/β2M.sup.−/PD-1.sup.− [0632] 3×KO (CD70): TRAC.sup.−/β2M.sup.−/CD70.sup.− [0633] 4×KO: TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−

[0634] Activated primary human T cells were electroporated with Cas9:gRNA RNP complexes. The nucleofection mix contained the Nucleofector™ Solution, 5×10.sup.6 cells, 1 μM Cas9, and 5 μM gRNA (as described in Hendel et al., *Nat Biotechnol.* 2015; 33(9):985-989, PMID: 26121415). For the generation of double knockout T cells (2×KO), the cells were electroporated with two different RNP complexes, each containing Cas9 protein and one of the following sgRNAs: TRAC (SEQ ID NO: 40) and β2M (SEQ ID NO: 41) at the concentrations indicated above. For the generation of triple knockout T cells (3×KO), the cells were electroporated with three different RNP complexes, each RNA complex containing Cas protein and one of the following sgRNAs: (a) TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), and PD-1 (SEQ ID NO: 42) at the concentrations indicated above; or (b) TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), and CD70 (SEQ ID NO: 36 or 37) at the concentrations indicated above. For the generation of quadruple knockout T cells (4×KO), the cells were electroporated with four different RNP complexes, each RNA complex containing Cas9 protein and one the following sgRNAs: TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), PD-1 (SEQ ID NO: 42), and CD70 (SEQ ID NO: 36 or 37) at the concentrations

indicated above. The unmodified versions (or other modified versions) of the gRNAs may also be used (e.g., SEQ ID NOS: 30, 31, 32, 26, and/or 27). Sequences in Tables 5 and 9.

TABLE-US-00009 TABLE 9 gRNA Sequences/Target Sequences Name Unmodified Sequence Modified Sequence TRAC sgRNA AGAGCAACAGUGCUGUGGCC A*G*A*GCAACAGUGCUGUGG guuuuagagcuagaaauagcaaguuaaaau CCguuuuagagcuagaaauagcaaguuaa aaggcuaguccguuaucaacuugaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 30) (SEQ ID NO: 40) TRAC sgRNA spacer AGAGCAACAGUGCUGUGGCC A*G*A*GCAACAGUGCUGUGG (SEQ ID NO: 98) CC (SEQ ID NO: 108) β2M sgRNA GCUACUCUCUCUUUCUGGCC G*C*U*ACUCUCUCUUUCUGG guuuuagagcuagaaauagcaaguuaaaau CCguuuuagagcuagaaauagcaaguuaa aaggcuaguccguuaucaacuugaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU aguggcaccgagucggugcU*U*U*U (SEQ ID NO: 31) (SEQ ID NO: 41) β2M sgRNA spacer GCUACUCUCUCUUUCUGGCC G*C*U*ACUCUCUCUUUCUGG (SEQ ID NO: 99) CC (SEQ ID NO: 109) PD-1 sgRNA CUGCAGCUUCUCCAACACAU C*U*G*CAGCUUCUCCAACAC guuuuagagcuagaaauagcaaguuaaaau AUguuuuagagcuagaaauagcaaguuaa aaggcuaguccguuaucaacuugaaaagu aauaaggcuaguccguuaucaacuugaaaa ggcaccgagucggugcUUUU (SEQ ID aguggcaccgagucggugcU*U*U*U NO: 32) (SEQ ID NO: 42) PD-1 sgRNA spacer CUGCAGCUUCUCCAACACAU C*U*G*CAGCUUCUCCAACAC (SEQ ID NO: 100) AU (SEQ ID NO: 110)

[0635] About one (1) week post electroporation, cells were either left untreated or treated with phorbol myristate acetate (PMA)/ionomycin overnight. The next day cells were processed for flow cytometry (see, e.g., Kalaitzidis D et al. J Clin Invest 2017; 127(4): 1405-1413) to assess TRAC, β2M, PD-1, and CD70 expression levels at the cell surface of the edited cell population. The following primary antibodies were used (Table 10):

TABLE-US-00010 TABLE 10 Antibodies Anti- body Clone Fluor Catalogue # Dilution For 1 TCR BW242/412 PE 130-091-236 (Miltenyi) 1:100 1 μL β2M 2M2 PE-Cy7 316318 (Biolegend) 1:100 1 μL PD-1 EH12.2H7 PE 329906 (Biolegend) 1:100 1 μL CD70 113-16 FITC 355105 (Biolegend) 1:100 1 μL

[0636] Tables 11 and 12 show highly efficient multiple gene editing. For the double-knock cells (2×KO; TRAC.sup.−/β2M.sup.−), 83% of viable cells lacked expression of TCR and β2M (Table 11; 3×KO (PD1)). For the triple knockout cells, 70% of viable cells lacked expression of TCR, β2M, and PD-1 (Table 11); and 80% of viable cells lacked expression of TCR, β2M, and CD70 irrespective of the CD70 gRNA used (Table 12). For the quadruple knockout cells (4×KO), 78% of viable cells lacked expression of TCR, β2M, PD-1, and CD70 (FIG. **1**).

TABLE-US-00011 TABLE 11 % of viable cells lacking expression in 2KO and 3KO (PD1) cell populations TRAC KO β2M KO PD1 KO 2 KO 3 KO (PD1) 2KO 98% 85% NA 83% NA 3 KO (PD1) 98% 73% 99% NA 70%

TABLE-US-00012 TABLE 12 % of viable cells lacking expression in 3KO (CD70) cell populations TRAC KO β2M KO CD70 KO 3KO (CD70) 3KO (CD70) (T7) 99% 79% 99% 80% 3KO (CD70) (T8) 99% 82% 99% 80%

[0637] To assess whether triple and quadruple gene editing in T cells affects cell expansion, cell numbers were enumerated among double, triple, and quadruple gene edited T cells (unedited T cells were used as a control) over a two week period of post editing. 5×10.sup.6 cells were generated and plated for each genotype of T cells.

[0638] As shown in FIG. **2**, cell proliferation (expansion) continued over the post-electroporation window test. Similar cell proliferation was observed among the double (β2M−/TRAC−), triple (β2M−/TRAC−/PD−1−, or β2M−/TRAC−/CD70−), and quadruple (β2M−/TRAC−/PD-1−/CD70)

knockout T cells, as indicated by the number of viable cells. These data suggest that multiple gene editing (up to triple and quadruple, with CD70 and PD-1 genes) does not impact T cell health as measured by T cell proliferation.

Example 3. Generation of CAR T Cells Lacking CD70 and/or PD1

Generation of Anti-CD70 CAR T Cells with Multiple Knockouts

[0639] This example describes the production of allogeneic human T cells that lack expression of the TCR gene, β2M gene, CD70 gene and/or PD1 gene, and express a chimeric antigen receptor (CAR) targeting CD70. These cells are designated TCR.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ or 3×KO (CD70) CD70 CAR.sup.+; TCR.sup.−/β2M.sup.−/PD1.sup.−/anti-CD70 CAR.sup.+ or 3×KO (PD1) CD70 CAR.sup.+; TCR.sup.−/β2M.sup.−/PD1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ or 4×KO CD70 CAR.sup.+ in the Figures.

[0640] A recombinant adeno-associated adenoviral vector, serotype 6 (AAV6) (MOI 50,000) comprising the nucleotide sequence of SEQ ID NO: 43 (comprising the donor template in SEQ ID NO: 44, encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 46) was delivered with Cas9:sgRNA RNPs (1 μM Cas9, 5 μM gRNA) to activated allogeneic human T cells. The following sgRNAs were used: TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), CD70 (SEQ ID NO: 36 or 37) and PD1 (SEQ ID NO: 42). The unmodified versions (or other modified versions) of the gRNAs may also be used (e.g., SEQ ID NOS: 30, 31, 32, 26, and/or 27). About one (1) week post electroporation, cells were processed for flow cytometry to assess TRAC, β2M, CD70, and PD1 expression levels at the cell surface of the edited cell population. The following primary antibodies were used (Table 13):

TABLE-US-00013 TABLE 13 Antibodies Antibody Clone Fluor Catalogue # Dilution TCR BW242/412 PE 130-091-236 (Miltenyi) 1:100 β2M 2M2 PE-Cy7 316318 (Biolegend) 1:100 CD70 113-16 FITC 355105 (Biolegend) 1:100 PD-1 EH12.2H7 PE 329906 (Biolegend) 1:100

[0641] T cell Proportion Assay. The proportions of CD4+ and CD8+ cells were then assessed in the edited T cell populations by flow cytometry using the following antibodies (Table 14):

TABLE-US-00014 TABLE 14 Antibodies Antibody Clone Fluor Catalogue # Dilution CD4 RPA-T4 BV510 300545 (Biolegend) 1:100 CD8 SK1 BV605 344741 (Biolegend) 1:100

High efficiency gene editing and CAR expression was achieved in the edited anti-CD70 CAR T cell populations. In addition, editing did not adversely alter CD4/CD8 T cell populations. FIG. **3** shows highly efficient gene editing and anti-CD70 CAR expression in the triple knockout CAR T cell. More than 55% of viable cells lacked expression of TCR, β2M, and CD70, and also expressed the anti-CD70 CAR. FIG. **4** shows that normal proportions of CD4/CD8 T cell subsets were maintained in the TRAC−/β2M−/CD70−/anti-CD70 CAR+ cells, suggesting that these multiple gene edits do not affect T cell biology as measured by the proportion of CD4/CD8 T cell subsets.

[0642] FIG. **5** shows show highly efficient gene editing and anti-CD70 CAR expression in the quadruple knockout CAR T cell. Greater than 60% of viable cells lacked expression of TCR, β2M, PD-1, and CD70, and expressed the anti-CD70 CAR. FIG. **6** shows that normal proportions of CD4/CD8 T cell subsets were maintained in the TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ cells, suggesting that these multiple gene editing do not affect T cell biology as measured by the proportion of CD4+/CD8+ T cell subsets.

Generation of Anti-BCMA CAR T Cells with Multiple Knockouts

[0643] This example describes the production of allogeneic human T cells that lack expression of the TCR gene, the β2M gene, the PD-1 gene, and/or the CD70 gene, and also express a chimeric antigen receptor (CAR) targeting B-cell maturation antigen (BCMA).

[0644] A recombinant adeno-associated adenoviral vector, serotype 6 (AAV6) comprising the nucleotide sequence of SEQ ID NO: 54 (comprising the donor template in SEQ ID NO: 55, encoding anti-BCMA CAR comprising the amino acid sequence of SEQ ID NO: 57) was delivered with Cas9:gRNA RNPs (1 μM Cas9, and 5 μM gRNA) to activated allogeneic human T cells. The following gRNAs were used: TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), PD-1 (SEQ ID

NO: 42), and CD70 (SEQ ID NO: 36 or 37). The unmodified versions (or other modified versions) of the gRNAs may also be used (e.g., SEQ ID NOS: 30, 31, 32, 26 and/or 27). About one (1) week post electroporation, cells were processed for flow cytometry as described above for anti-CD70 CAR+ T cells, with the following difference. Anti-BCMA CAR expression was detected using biotinylated recombinant human BCMA (ACROS Cat #BC7-H82F0). The double and quadruple knockout anti-BCMA CAR.sup.+ cells were then characterized as described herein.

[0645] FIGS. **7**A-**7**B shows highly efficient gene editing of the TRAC gene, β2M gene, the CD70 gene and the PD-1 gene. FIG. **7**C shows high expression of the anti-BCMA CAR+ cells in double knockout and quadruple knockout cells.

Generation of Anti-CD19 CAR T Cells with Multiple Knockouts

[0646] Allogeneic human T cells were generated that express a chimeric antigen receptor (CAR) targeting CD19 and lack the expression of the TCR gene, the ββ2M gene, and optionally the CD70 gene.

[0647] To generate the allogeneic T cells, activated primary human T cells were electroporated with Cas9:gRNA RNP complexes and infected with adeno-associated adenoviral vectors (AAVs) containing anti-CD19 CAR donor template with homology to the TRAC locus. Recombinant AAV serotype 6 (AAV6) comprising the nucleotide sequence of SEQ ID NO: 155 (comprising the donor template in SEQ ID NO: 156, encoding anti-CD19 CAR comprising the amino acid sequence of SEQ ID NO: 149) was delivered with Cas9:sgRNA RNPs (1 µM Cas9, 5 µM gRNA) to activated human T cells. The following sgRNAs were used to knock-out the respective genes: TRAC (SEQ ID NO: 40), 02M (SEQ ID NO: 41), CD70 (SEQ ID NO: 36). The unmodified versions (or other modified versions) of the gRNAs may also be used (e.g., SEQ ID NOs: 30, 21 or 27). About one (1) week post electroporation, cells were processed for flow cytometry as described above for anti-CD70 CAR+ T cells, with the following difference. Anti-CD19 CAR expression was detected using biotinylated recombinant human CD19 (ACROBIOSYSTEMS INC; CD9-H825). The CD70 deficient anti-CD19 CAR.sup.+ T cells were then characterized as described herein.

Generation of Anti-CD33 CAR T Cells with Multiple Knockouts

[0648] Allogeneic human T cells were generated that express a chimeric antigen receptor (CAR) targeting CD33 and lack the expression of the T cell receptor (TCR) gene (gene edited in the TCR Alpha Constant (TRAC) region), the β2-microglobulin (β2M) gene, and optionally the CD70 gene.

[0649] To generate the allogeneic T cells, activated primary human T cells were electroporated with Cas9:gRNA RNP complexes and infected with adeno-associated adenoviral vectors (AAVs) containing anti-CD33 CAR donor template with homology to the TRAC locus. Recombinant AAV serotype 6 (AAV6) comprising the nucleotide sequence of SEQ ID NO: 87 (comprising the donor template in SEQ ID NO: 135, encoding anti-CD33 CAR comprising the amino acid sequence of SEQ ID NO: 139 was delivered with Cas9:sgRNA RNPs (1 µM Cas9, 5 µM gRNA) to activated human T cells. The following sgRNAs were used to knock-out the respective genes: TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), CD70 (SEQ ID NO: 36). The unmodified versions (or other modified versions) of the gRNAs may also be used (e.g., SEQ ID NOs: 30, 21 or 27).

[0650] Populations of TCR+ T cells (no RNP) and TRAC−/β2M− T cells (TCR and β2M deficient cells without a CAR) were similarly generated for use as controls. About one (1) week post electroporation, cells were processed for flow cytometry as described above for anti-CD70 CAR+ T cells, with the following difference. Anti-CD33 CAR expression was detected using biotinylated recombinant human CD33 (data not shown). The CD70 knockout anti-CD33 CAR.sup.+ T cells were then characterized as described herein.

Characterization of CD4/CD8 Cell Populations in Anti-CD33 CAR T Cells with CD70 Knock-Out

[0651] CD33 can be expressed on T cells with higher levels observed on cultured CD4 cells than CD8 cells. During the course of producing anti-CD33 CAR-T cells CD4 cells become substantially reduced due to fratricide. As shown in FIG. **8**, anti-CD33 CAR-T cell cultures with intact CD70 displayed a 97% reduction in CD4+ cells over a 3 week culture period, while cultures of cells with

disrupted CD70 showed only a 61% reduction over this time course. Thus disrupting the CD70 gene appears to reduce the fratricide observed in the anti-CD33 CAR-T cell cultures. Without wishing to be bound by theory, this effect may occur through an immune stimulatory function which could be potentiated by CD70/CD27 interactions, and genetic disruption of CD70 results in more balanced CD4/CD8 ratios that may be more optimal for therapeutic benefit in malignancy.

Example 4: CD70 KO Improves Cell Proliferation

Effect of CD70 KO on Cell Proliferation of Anti-CD33 CAR T Cells In Vitro To assess the ability of cells to expand in cytokine containing media (IL-2+IL-7), anti-CD33 CAR T cells were utilized. Specifically, $5\times10.\sup.6$ total anti-CD33 CAR T cells comprising a double knockout (TRAC−/B2M−) or triple knockout (TRAC−/B2M−/CD70−) were generated as described in Example 3, plated and allowed to grow in a 10 mL volume of cytokine containing media. After 1 week cells were counted. $5\times10.\sup.6$ cells from the previous culture were then replated in 10 mL volume (fresh cytokine containing media) and 1 week later the total number of cells were enumerated. Allogeneic anti-CD33 CAR-T cells containing a disruption in the CD70 gene expanded to greater levels on the first and second week of replating (FIG. **9**). These data show that CD70 knockout can result in greater cell yields in culture.

Effect of CD70 KO on Cell Proliferation of Anti-CD19 CAR T Cells In Vitro

[0652] To further assess the ability of cells to expand in cytokine containing media (IL-2+IL-7), anti-CD19 CAR T cells were utilized. Specifically, $5\times10.\sup.6$ total anti-CD19 CAR T cells comprising a double knockout (TRAC−/B2M−) or triple knockout (TRAC−/B2M−/CD70−) were generated as described in Example 3, plated and allowed to grow in a 10 mL volume of cytokine containing media. After 1 week cells were counted. $5\times10.\sup.6$ cells from the previous culture were then replated in 10 mL volume (fresh cytokine containing media) and 1 week later the total number of cells were enumerated. Allogeneic anti-CD19 CAR-T cells containing a disruption in the CD70 gene expanded to greater levels on the first and second week of replating as compared to control cells without a CD70 gene disruption (FIG. **10**). These data show that CD70 knockout can result in greater cell yields in culture.

Effect of CD70 KO on Cytokine Driven Proliferation and Apoptosis of Anti-BCMA CAR T Cells In Vitro

[0653] Cytokine driven proliferation. To evaluate the effect of CD70 and/or PD1 knockout on cell proliferation, anti-BCMA CAR T cells were utilized. Anti-BCMA CAR T cells were generated as described in Example 3. The following groups of edited T cells were generated: [0654] TRAC−/B2M−/anti-BCMA CAR+ (Control; 2KO, BCMA CAR+) [0655] TRAC−/B2M−/CD70−/anti-BCMA CAR+ (3KO (CD70), BCMA CAR+) [0656] TRAC−/B2M−/PD1−/anti-BCMA CAR+ (3KO (PD1), BCMA CAR+) [0657] TRAC−/B2M−/CD70−/PD1−/anti-BCMA CAR+ (4KO, BCMA CAR+)

[0658] Edited cells were enriched for TRAC−/B2M− cells by magnetic depletion of CD3+B2M+ cells. Briefly, cells were labelled with anti-CD3 Biotin (Biolegend Cat #300404) anti-β2M Biotin (Biolegend Cat #316308) antibodies, each at 0.5 µg per $1\times10.\sup.6$ cells in 100 µl volume at 4° C. for 15 min, washed and incubated with Streptavidin labelled magnetic microbeads (Miltenyi Biotech, 130-048-101) for 15 min at 4° C. Cells were resuspended in buffer and passed through LS columns (Miltenyi Biotech, 130-042-401) according to the manufacturer's protocol. To determine the effect of CD70 or PD1 on IL-2/IL-7 driven T cell proliferation, the edited T cells (1E6 cells/ml) were cultured in growth medium (X-vivo medium (04-744, Lonza), supplemented with 5% human AB serum (HP1022, Valley Biomedical)), 50 ng/ml IL-2 (rhIL-2; 130-097-745, Miltenyi Biotech) and 10 ng/ml IL-7 (rhIL-7; Cellgenix 001410-050) for up to four weeks. At indicated days, the cells were counted and re-seeded in fresh medium at 1.5E6 cells/ml in appropriate culture dishes.

[0659] FIGS. **11** and **12** show that knockout of CD70 improved IL-2/IL-7 driven proliferation of anti-BCMA CAR T cells in vitro, as compared to CD70 sufficient controls (i.e. anti-BCMA CAR T cells comprising endogenous CD70). FIG. **11** also shows the CD70 KO can improve health and

proliferation competence of anti-BCMA CAR T cells even when the T cells from this donor appear to be in significant decline after 17 days when the CD70 gene is intact (as shown by the reduced cell numbers of donor 1 (FIG. **11**) compared to donor 2 (FIG. **12**)). This property of maintaining T cell health (enabled by KO of the CD70 gene) is broadly applicable to many aspects of CAR T development including: extended expansion during manufacturing increasing yield and consistency, rescue of exhausted/unhealthy T cells enabling potentially lower doses in patients and more robust responses, combination with other KOs that may be more detrimental to T cell health but have other advantages such as overcoming suppression of T cell activity (e.g. PD1 KO). As shown in FIGS. **11** and **12**, deleting the PD1 gene by itself shows no benefit to CAR T cell expansion but when combined with a CD70 KO shows synergistic effects.

[0660] Apoptosis. The effect of CD70 KO on apoptotic cell death of anti-BCMA CAR+ T cells following exposure to antigen was evaluated in an antigen rechallenge assay. Briefly, to achieve antigen exposure, anti-BCMA CAR+ T cells were exposed to plate-adhered recombinant BCMA protein. Plates with adhered antigen were prepared by coating 24 well plates with recombinant BCMA protein in 1×PBS (1 µg/ml; biotinylated Human BCMA Protein, ACRO Biosystems) overnight at 4° C. and then washing away unbound antigen. Following the wash, antigen-bound plates were then used to challenge anti-BCMA CAR+ T cells either with or without a CD70 knockout. The 2×KO (TRAC.sup.−/B2M−) anti-BCMA CAR+ T cells and 3×KO (TRAC−/B2M−/CD70−) anti-BCMA CAR+ T cells (1×10.sup.6 cells/ml) were exposed to plate-bound recombinant BCMA protein (1 µg/ml) for 24 hours in growth medium (X-vivo medium (04-744, Lonza), 5% human AB serum (HP1022, Valley Biomedical)) supplemented with IL-2 (rhIL-2; 130-097-745, Miltenyi Biotech). Cells were then washed, counted and re-challenged (1×10.sup.6 cells/ml) with fresh plate-bound antigen every 24 hours for a total of three consecutive re-challenges (24 hr, 48 hr, and 72 hr). At the end of each re-challenge, an aliquot of cells was washed and stained with fluorochrome-conjugated annexin V along with propidium iodide in annexin V binding buffer (BioLegend) for 15 minutes at room temperature. Cells were then washed and resuspend in annexin V binding buffer for analysis by flow cytometry. The cells were counted at each time point and the cell count per ml was derived. For the calculation of fold-expansion at each time point, the initial fold-expansion at time 0 was set at 1. Fold-expansion for all other time points were calculated by multiplying the cell count per ml at each time point by the fold-expansion per ml for the prior time point. For example, the fold expansion at 72 hr was calculated by multiplying the cell count per ml at 72 hr by the fold expansion per ml at 48 hr.

[0661] FIG. **13** demonstrates that the deletion of CD70 (CD70 KO) rescues anti-BCMA CAR+ T cells from apoptosis, as shown by the decrease in the percentage of apoptotic cells following the second (48 hr) and third (72 hr) rechallenge. Furthermore, the absence of CD70 expression in anti-BCMA CAR+ T cells surprisingly enhances the expansion of the anti-BCMA CAR+ T cells in response to antigen exposure (FIG. **14**).

Effect of CD70 KO on Cell Proliferation of Anti-CD70 CAR T Cells In Vitro

[0662] To further assess the impact of disrupting the CD70 gene in CAR T cells, anti-CD70 CAR T cells were generated as described in Example 3. Specifically, 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR T cells were generated using two different gRNAs (T7 (SEQ ID NO: 36 and T8 (SEQ ID NO: 37)). After electroporation, cell expansion was assessed as described in Example 2 by counting viable cells. FIG. **15** shows that triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells generated with either T7 or T8 gRNAs exhibited greater cell expansion relative to double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells. These data suggest that knocking-out the CD70 gene gives a cell proliferation advantage to anti-CD70 CAR+ T cells.

[0663] Cell expansion was also assessed in the quadruple knockout, TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells. These cells exhibited greater expansion relative to triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells and to double

knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells (FIG. **16**).

Example 5. CD70 KO Increases Durability and Potency of CAR T Cells In Vitro

Cell Killing Function of Anti-CD19 CAR T Cells with CD70 Knock-Out

[0664] Following preparation of edited anti-CD19 CAR T cells as described in Example 3, the functional activity of the CAR T cells was verified using a flow cytometry-based cytotoxicity assay. The anti-CD19 CAR T cells (TRAC−/β2M−/CD19 CAR+ and TRAC−/β2M−/CD70−/CD19 CAR+) were co-cultured with one of two CD19-expressing cancer cell lines (target cells): Nalm6 (ATCC cr13273) or Raji (ATCC ccl-86). The target cells were labeled with 5 μM efluor670 (eBiosciences), washed and incubated in co-cultures with the TRAC−/β2M−/anti-CD19 CAR+, or TRAC−/β2M−/CD70−/anti-CD19 CAR+ at varying ratios (0.01, 0.05, 0.1, 0.5, 1:1 T cells:target cells). The target cells were seeded at 50,000 cells per well in a 96-well, U-bottom plate. The co-culture was incubated overnight. After incubation, wells were washed and media was replaced with 200 μL of media containing a 1:500 dilution of 5 mg/mL DAPI (Molecular Probes). 25 μL of CountBright beads (Life Technologies) were then added to each well and the cell cultures were analyzed for cell viability by flow cytometry (i.e., viable cells being negative for DAPI staining).

[0665] Percent cell lysis of the target cells (e.g.: Nalm6 or Raji cells) was then determined using the following formula:

Percent cell lysis=(1−((total number of target cells in a test sample)÷(total number of target cells in a control sample))×100; [0666] wherein a test sample was target cells (e.g.: Nalm6 or Raji cells) co-cultured with 1) TRAC−/β2M−/CD19 CAR+ T cells or 2) TRAC−/β2M−/CD70−/CD19 CAR+ T cells; and [0667] a control sample was target cells alone that had not been co-cultured.

[0668] Disruption of the CD70 gene led to enhanced cytolytic activity of the anti-CD19 CAR-T cells against the Raji cell line at low CAR-T to target ratios (FIG. **17**, bottom panel). Disruption of CD70 did not enhance anti-CD19 CAR-T activity against the Nalm6 cell line (FIG. **17**, top panel). Of note, the Nalm6 cell line is relatively easier to lyse by these CAR-T cells (>80% lyses at 0.1:1 CAR-T cell to target ratio for Nalm6 vs 0% for Raji at this ratio for the wild-type cells) likely explaining the lack of resulting increased efficacy due to CD70 disruption in this assay against Nalm6 cells. The increased activity conferred by CD70 loss against the Raji cell line indicates that in challenging tumor environments, particularly when CAR-T to tumor ratios are low, CD70 loss may have substantial benefit to the CAR-T cells in eradicating tumor cells.

Cell Killing Function of Anti-CD33 CAR T Cells with CD70 Knock-Out

[0669] Following preparation of the edited anti-CD33 CAR+ T cells as described in Example 3, the functional activity of the CAR T cells was verified using a flow cytometry-based cytotoxicity assay. The anti-CD33 CAR T cells (TRAC−/β2M−/CD33 CAR+ and TRAC−/β2M−/CD70−/CD33 CAR+) or control T cells (no RNP) were co-cultured with the CD33-expressing cancer cell line MV4-11 (ATCC CRL-9591). The target cells were labeled with 5 μM efluor670 (eBiosciences), washed and incubated in co-cultures with the TRAC−/B2M−/anti-CD33 CAR+, TRAC−/β2M−/CD70−/anti-CD33 CAR+, or controls at varying ratios (0.01:1, 0.03:1, 0.06:1, 0.125:1, 0.25:1, 0.5:1, or 1:1 T cells:target cells). The target cells were seeded at 50,000 cells per well in a 96-well, U-bottom plate. The co-culture was incubated overnight. After 48 hrs, wells were washed and media was replaced with 200 μL of media containing a 1:500 dilution of 5 mg/mL DAPI (Molecular Probes). 25 μL of CountBright beads (Life Technologies) were then added to each well and the cell cultures were analyzed for cell viability by flow cytometry (i.e., viable cells being negative for DAPI staining).

[0670] Percent cell lysis of the target cells (e.g.: MV4-11) was then determined using the following formula:

Percent cell lysis=(1−((total number of target cells in a test sample)+(total number of target cells in a control sample))×100; [0671] wherein a test sample was target cells (e.g.: MV4-11 cells) co-

cultured with 1) TRAC−/B2M−/CD33 CAR+ T cells; or 2) TRAC−/B2M−/CD70−/CD33 CAR+ T cells, and [0672] a control sample was target cells alone that had not been co-cultured.

[0673] Although both populations of anti-CD33 CAR T cells effectively killed MV4-11 cells, reaching nearly 100% cells kill at ratios of 0.5 CAR T cell: MV4-11 cell, the TRAC−/B2M−/CD70−/CD33 CAR+ T cells demonstrated higher cell killing at lower CAR T to cancer cell rations (FIG. **18**). These data demonstrate that allogeneic anti-CD33 CAR T cells with the additional CD70 knock-out are more efficacious at lower CAR T cell to target cell ratios.

Cell Killing Function of Anti-CD70 CAR T Cells with CD70 Knock-Out

[0674] A cell killing assay was used to assess the ability of the TRAC$^{−}$/β2M$^{−}$/CD70$^{−}$/anti-CD70 CAR^{+} cells and TRAC$^{−}$/β2M$^{−}$/PD-1$^{−}$/CD70$^{−}$/anti-CD70 CAR^{+} cells to kill a CD70^{+} adherent renal cell carcinoma (RCC)-derived cell line (A498 cells). Adherent cells were seeded in 96-well plates at 50,000 cells per well and left overnight at 37° C. The next day edited anti-CD70 CAR T cells were added to the wells containing target cells at the indicated ratios. After the indicated incubation period, CAR T cells were removed from the culture by aspiration and 100 μL Cell titer-Glo (Promega) was added to each well of the plate to assess the number of remaining viable cells. The amount of light emitted per well was then quantified using a plate reader. The cells exhibited potent cell killing of RCC-derived cells following 24-hour co-incubation (FIG. **19**). The anti-CD70 CAR T cells demonstrated higher potency when CD70 was knocked out, which is clearly visible at low T cell: A498 ratios (1:1 and 0.5:1) where cell lysis remains above 90% for TRAC$^{−}$/β2M$^{−}$/CD70$^{−}$/anti-CD70 CAR^{+}, while cells lysis drops below 90% for the TRAC$^{−}$/β2M$^{−}$/anti-CD70 CAR^{+}. This suggests that knocking-out the CD70 gene gives a higher cell kill potency to anti-CD70 CAR+ T cells.

Example 6. Rechallenge of CD70 Deficient CAR T Cells In Vitro

CD70 Knockout Improves Anti-CD33 CAR+ T Cell Killing Upon Serial Rechallenge

[0675] To assess the ability of cells to expand after challenge and rechallenge with antigen-expressing cells (e.g.: target cells) anti-CD33 CAR T cells were generated as described in Example 3 and utilized. Specifically, $5×10^6$ total T cells were plated in the presence of $5×10^6$ irradiated target cells (MV-4-11) and allowed to grow in a 10 mL volume. After 1 week, cells were counted, $5×10^6$ cells from the previous culture were then replated in 10 mL volume along with a fresh aliquot of $5×10^6$ irradiated target cells and 1 week later the total number of cells were enumerated. The process was repeated as indicated, each rechallenge started with $5×10^6$ cells. The number of viable cells were counted as described in Example 2. Allogeneic anti-CD33 CAR-T cells containing a disruption in the CD70 gene expanded to greater levels on the second week of after 2 challenges with MV-4-11 cells (FIG. **20**). These data show that CD70 can limit T-cell expansion in the presence of antigen expressing cells and its loss can result in greater cell expansion after antigen stimulation.

CD70 Knockout Improves Anti-CD19 CAR+ T Cell Killing Upon Serial Rechallenge

[0676] To further assess the ability of cells to expand after challenge and rechallenge with antigen-expressing cells (e.g.: target cells) anti-CD19 CAR T cells were generated as described in Example 3 and utilized. Specifically, $5×10^6$ total T cells were plated in the presence of $5×10^6$ irradiated target cells (Nalm6) and allowed to grow in a 10 mL volume. After 1 week, cells were counted, $5×10^6$ cells from the previous culture were then replated in 10 mL volume along with a fresh aliquot of $5×10^6$ irradiated target cells and 1 week later the total number of cells were enumerated. The process was repeated as indicated, each rechallenge started with $5×10^6$ cells. The number of viable cells were counted as described in Example 2. Allogeneic anti-CD19 CAR-T cells containing a disruption in the CD70 gene expanded to similar amounts during the first 2 challenges. However, at three challenges allogeneic anti-CD19 CAR-T cells containing a disruption in the CD70 gene expanded to greater level on the third challenge with Nalm6 cells (FIG. **21**). These data show that the presence of CD70 can limit T-cell expansion in the presence of antigen

expressing cells and its loss can result in greater cell expansion after repeated antigen stimulation.

Knockout of CD70, or PD-1 Plus CD70, Maintain Anti-CD70 CAR.SUP.+ T Cell Killing Upon Serial Rechallenge

[0677] The anti-CD70 CAR.sup.+ T cells generated above were serially rechallenged with CD70+ kidney cancer cell line, A498, and evaluated for their ability to kill the CD70+ kidney cancer cell lines A498 or ACHN.

[0678] A498 cells were plated in a T25 flask and mixed at a ratio of 2:1 (T-cell to A498) with $10 \times 10.sup.6$ anti-CD70 CAR.sup.+ T cells containing either two (TRAC.sup.−/β2M.sup.−), three (TRAC.sup.−/β2M.sup.−/PD-1.sup.−) or (TRAC.sup.−/β2M.sup.−/CD70.sup.−)), or four (TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−) gRNA edits.

[0679] Two or three days after each challenge, cells were counted, washed, resuspended in fresh T cell media, and re-challenged the next day with the same ratio of two anti-CD70 CAR.sup.+ T cell per one A498 cell (2:1, CAR.sup.+ T:target). Challenging of anti-CD70 CAR.sup.+ T cells with CD70+ A498 cells was repeated 13 times. Three to four days following each exposure to A498 cells (and prior to the next rechallenge), aliquots of the culture were taken and analyzed for the ability of the CAR T Cells to kill A498 or ACHN target cells at a ratio of 2:1 (CAR T cell:Target cell). Cell kill was measured using Cell titer-glo (Promega). Prior to the first challenge with A498, anti-CD70 CAR+ T cells with 2×KO (TRAC.sup.−/β2M.sup.−), 3×KO (TRAC.sup.−/β2M.sup.−/CD70), 3×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−), and 4×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−) each exhibited a target cell killing of A498 cells approaching 100%. By challenge nine however, the 2×KO (TRAC.sup.−/β2M.sup.−) and 3×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−) anti-CD70 CAR.sup.+ T cells induced target cell killing of A498 cells below 40%, while 3×KO (TRAC.sup.−/β2M.sup.−/CD70) and 4×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−) anti-CD70 CAR.sup.+ T cells exhibited target cell killing above 60% (FIG. **22**A). The target cell killing for 3×KO (TRAC.sup.−/β2M.sup.−/CD70) and 4×KO (TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−) anti-CD70 CAR.sup.+ T cells remained above 60% even following 13 re-challenges with A498 cells, demonstrating that these CAR+ T cells were resistant to exhaustion.

[0680] Anti-CD70 CAR T cells were also evaluated for their ability to kill ACHN cells at a ratio of 2:1 (T-cell to ACHN) following serial rechallenge with A498 renal carcinoma cells (FIG. **22**B). Prior to the first challenge with A498, the double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells, the triple knockout TRAC.sup.−/β2M.sup.−/PD-1-anti-CD70 CAR.sup.+ T, the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and the quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T exhibited a cell kill efficiency above 62%, 47%, 73% and 81%, respectively.

[0681] After challenge five, the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and the quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup. −/anti-CD70 CAR.sup.+ T cells still efficiently killed above 55% of ACHN cells at a ratio of 2:1 (T-cell to ACHN), while the double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells and the triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cell kill dropped below 11% of ACHN cells. This trend continued, wherein the double knockout TRAC.sup. −/β2M.sup.−/anti-CD70 CAR.sup.+ T cells and the triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells failed to survive beyond 10 rechallenges. In contrast, the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and the quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells continued to expand in culture and to kill greater than 30% of ACHN cells at a ratio of 2:1 (T-cell to ACHN) following two rechallenges.

[0682] The data demonstrate that the 4×KO, CD70 CAR.sup.+ T cells and the 3×KO (CD70), CD70 CAR.sup.+ cells are more potent than the 2×KO, CD70 CAR.sup.+ T or 3×KO (PD1), CD70 CAR.sup.+ T cells. In addition, the 3×(CD70) KO and 4×KO prevents T cell exhaustion.

[0683] After 5 rechallenges the cells were evaluated for their ability to kill cancer cells. Surprisingly, the 3KO and 4KO anti-CD70 CAR+ T cells remained highly effective at killing cancer cells (FIG. **23**A) even after multiple cancer cell challenges. The cell killing effect of the anti-CD70 CAR+ T cells on ACHN cells is reproducible at even at reduced effector to target cell ratios of 1:1, 0.5:1, and 0.25:1. (FIG. **23**A).

[0684] To ensure long-term benefit upon CAR T treatment, CAR T cells should be able to identify and eradicate their target cells over a long period of time, to rule out the possibility of cancer cell escape from CAR-T mediated cell kill. The in vitro re-challenge assay mimics a recurrent encounter of CAR-T cells with target cells over several cycles of CAR-T cell activation. These data demonstrate the superiority of the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and of the quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells, in sustaining multiple challenges with kidney cancer cells, without showing reduction of their target cell killing ability, as compared to the double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells and the triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells.

[0685] Exhaustion and activation markers were also measured by flow cytometry in the anti-CD70 CAR+T cells following rechallenge. After 8 challenges, the Triple (TRAC−/β2M−/CD70−) and Quadruple (TRAC−/β2M−/PD1−/CD70−) KO anti-CD70 CAR+ T cells exhibited higher activation marker LAG3 expression than the Double (TRAC−/β2M−) and Quadruple (TRAC−/β2M−/PD1−/CD70−) KO anti-CD70 CAR+ T cells, consistent with their level of high cell kill activity. It was observed that PD1 expression was lower in the Triple (TRAC−/β2M−/CD70−) anti-CD70 CAR+T cells (similar to Triple (TRAC−/β2M−/PD1−) and Quadruple (TRAC−/β2M−/PD1−/CD70−) KO anti-CD70 CAR+T cells) compared to the Double (TRAC−/β2M−) anti-CD70 CAR+T cells, suggesting that knocking-out CD70 has an effect on the downregulation of the exhaustion marker PD1 expression in the Anti-CD70 CAR+T cells. (FIG. **23**B).

Knockout of PD-1 and CD70 Maintains Anti-BCMA CAR.SUP.+ T Cell Killing Upon Serial Rechallenge

[0686] The anti-BCMA CAR.sup.+ T cells generated as described in Example 3 were serially rechallenged with and evaluated for their ability to kill the BCMA+ multiple myeloma cell line MM.1S (ATCC CRL-2974). The ability to secrete cytokines upon serial T cell activation through CAR engagement was also measured after each rechallenge. MM.1S cells were labeled with 5 μM eFlour670 and mixed at a ratio of 2:1 (MM.1S to T-cell) in a 6 well tissue culture dish with 1×10.sup.6 anti-BCMA CAR.sup.+ T cells containing either two (TRAC.sup.−/β2M.sup.−) or four ((TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−) gRNA edits. One day following exposure to MM.1S cells, an aliquot of the culture was taken and analyzed for both target cell kill & IFN-g secretion by CAR-T cells. To measure cytokine release, T cells and target cells were co-incubated for 24 hours at the ratios indicated. Supernatant media was collected for use in IL-2 or IFNγ ELISAs (RD Systems) on a new plate following the manufacturer's instructions (RD Systems). To quantify cell killing, cells were washed, media was replaced with 200 mL of media containing a 1:500 dilution of 5 mg/mL DAPI (Molecular Probes) (to enumerate dead/dying cells). Finally, 25 mL of CountBright beads (Life Technologies) was added to each well. Cells were then processed by flow cytometry. [0687] 1) Cells/mL=((number of live target cell events)/(number of bead events))×((Assigned bead count of lot (beads/50 μL))/(volume of sample)) [0688] 2) Total target cells were calculated by multiplying cells/mL×the total volume of cells. [0689] 3) The percent cell lysis was then calculated with the following equation:

% Cell lysis=(1−((Total Number of Target Cells in Test Sample)/(Total Number of Target Cells in Control Sample))×100

[0690] Two or three days after each challenge, cells were counted, washed, resuspended in fresh T cell media, and rechallenged with the same ratio of one anti-BCMA CAR.sup.+ T cell per two

eFlour670 labeled MM.1S cells. Challenging of anti-BCMA CAR.sup.+ T cells with BCMA+ MM.1S cells was repeated 10 sequential times. Prior to any challenge with MM.1S cells, co-incubation of either 2×KO (TRAC−/B2M−) or 4×KO (TRAC−/B2M−/CD70−/PD-1−) anti-BCMA CAR+ T cells with MM.1S cells resulted in complete killing of target cells. Additionally, IFNγ production by both 2×KO and 4×KO anti-BCMA CAR+ T cells was similar. Following a 4th rechallenge with MM.1S cells however, target cell killing and IFNγ production by 2×KO anti-BCMA CAR+ T cells decreased relative to that induced by 4×KO anti-BCMA CAR+ T cells. By the 8th rechallenge, target cell killing was only approximately 20% for 2×KO anti-BCMA CAR+ T cells, while both IFNg and target cell killing by 4×KO anti-BCMA CAR+ T cells remained comparable to that seen prior to any challenge with MM.1S cells (FIGS. **24**A-**24**B). In addition, the quadruple knockout anti-BCMA CAR T cells showed higher proliferation in response to exposure to target cells (FIG. **24**C).

[0691] To ensure long-term benefit upon CAR T treatment, CAR T cells should be able to identify and eradicate their target cells over long period of time, to rule out the possibility of cancer cell escape from CAR-T mediated cell killing. The in vitro rechallenge assay mimics a recurrent encounter of CAR-T cells with target cells over several cycles of CAR-T cell activation, therefore demonstrating the superiority of the 4× knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-BCMA CAR.sup.+ T cells, in sustaining multiple challenges with target cells, without showing reduction of cell killing ability, as compared to the double knockout TRAC.sup.−/β2M.sup.−/anti-BCMA CAR.sup.+ T cells (FIGS. **24**A-**24**C).

Example 7. CD70 KO Overcomes Challenge of Excess Inhibitory Molecules

Comparison of the Effects of Multi-Knockout Anti-CD70 CAR+ T Cells on A498-PD-L1 Renal Carcinoma Cells

[0692] Cell Kill Assay. The ability of multi-gene edited anti-CD70 CAR.sup.+ cells to kill A498 renal carcinoma cells overexpressing PD-L1 was determined using the cell kill assay described herein. To create cells overexpressing PD-L1 (CD274), A498 cells were infected with lentivirus encoding a PD-L1 cDNA and a puromycin resistance gene (Genecopoeia). After selection with puromycin, cells were stained with an anti-PD-L1 antibody to assess expression of PD-L1. The A498 cells expressing PD-L1 are referred to as A498-PD-L1 and were used in the functional assays described.

[0693] The TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ (2×KO, CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ (3×KO (PD-1), CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (3×KO (CD70), CD70 CAR.sup.+) and TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (4×KO, CD70 CAR.sup.+) T cells were incubated with the A498-PD-L1 cells at a CAR T cell:A498-PD-L1 target cells ratio of 2:1 (FIG. **25**A), 1:1 (FIG. **25**B), or 0.5:1 (FIG. **25**C). The CD70 knockout cells exhibited potent cell killing of RCC-derived cells following 24-hour co-incubation (FIGS. **25**A-**25**C). The cells with PD1 knockout alone did not effectively lyse cells in the presence of PD-L1 overexpression. However, the CD70 knockout was able to rescue the PD1 knockout and enhanced cell lysis was observed in the CART cells with CD70 KO and PD1 KO. These data demonstrate that the loss of CD70 on the surface of these CAR-T cells enhances their function even in the presence of highly immune suppressive molecules expressed by tumor cells such as PD-L1.

[0694] Cytokine Release Assay. A cytokine release assay was performed as described herein. The ability of the double knockout, triple knockout, and quadruple knockout anti-CD70 CAR.sup.+ T cells to produce IL-2 and IFN-g when co-cultured in the presence of A948-PD-L1 cells following 24-hour co-incubation at a ratio (CAR T cell:A948-PD-L1 target cell) of 1:1 was assessed using an ELISA assay. IL-2 and IFN-g from supernatants of cell co-cultures were measured. The quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells secreted the highest levels of IFN-g (FIG. **26**A) and IL-2 (FIG. **26**B) when cultured with A948-PD-L1 cells. These data demonstrate the knock-out of CD70 enhances CAR-T cells secretion of cytokines even

in the presence of highly immune suppressive molecules expressed by tumor cells such as PD-L1. The knockout of CD70 together with a knockout of PD-1 in CAR-T cells further enhances the effect. Without wishing to be bound by theory, it is believed knocking-out CD70 in anti-CD70 CAR+ T cells can rescue the detrimental phenotypes of other cell knockouts (e.g.: PD1). These data demonstrate that knocking-out CD70 in CAR T cells enhances target cell killing and CAR T cell function in a highly immune suppressive context.

Example 8. CD70 Knockout Improves In Vivo Efficacy

Efficacy of CD70 and PD1 Knockout in Anti-CD70 CART Cells: The Subcutaneous Renal Cell Carcinoma Tumor Xenograft Model in NOG Mice

Treatment in Small Tumor Model

[0695] The ability of T cells expressing a CD70 CAR to eliminate kidney carcinoma cells that express high levels of CD70 was evaluated in in vivo using a subcutaneous renal cell carcinoma (A498) tumor xenograft model in mice.

[0696] CRISPR/Cas9 and AAV6 were used as above (see for example, Example 3) to create human T cells that lack expression of the TCR, β2M, CD70 and/or PD1 with concomitant expression from the TRAC locus using a CAR construct targeting CD70 (SEQ ID NO: 45; SEQ ID NO: 46). In this example activated T cells were first electroporated with 2, 3 or 4 distinct Cas9:sgRNA RNP complexes containing sgRNAs targeting TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), PD1 (SEQ ID NO: 42), and CD70 (SEQ ID NO: 36 or 37). The DNA double stranded break at the TRAC locus was repaired by homology directed repair with an AAV6-delivered DNA template comprising a donor template (SEQ ID NO: 44; SEQ ID NO: 45) (encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 45) containing right and left homology arms to the TRAC locus flanking a chimeric antigen receptor cassette (−/+ regulatory elements for gene expression).

[0697] The resulting modified T cells are 2×KO (TRAC−/β2M−), 3×KO (TRAC−/β2M−/PD1− or TRAC−/β2M−/CD70−) and 4×KO (TRAC−/β2M−/PD1−/CD70−) anti-CD70 CAR+ (with 41BB costimulatory domain) T cells. The ability of these anti-CD70 CAR+ T cells to ameliorate disease caused by a CD70+ renal carcinoma cell line was evaluated in NOG mice using methods employed by Translational Drug Development, LLC (Scottsdale, AZ). In brief, 12, 5-8 week old female, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. Mice received a subcutaneous inoculation of $5×10.sup.6$ A498 renal carcinoma cells/mouse in the right hind flank. When mean tumor size reached 25-75 mm.sup.3 (target of ˜50 mm.sup.3), the mice were further divided into 5 treatment groups as shown in Table 15. On Day 1, treatment group 2 to 5 received a single 200 µl intravenous dose of anti-CD70 CAR+ T cells according to Table 15.

TABLE-US-00015 TABLE 15 Treatment groups T cell treatment Group CAR-T A498 cells (i.v.) N 1 None 5 × 10.sup.6 None 5 cells/mouse 2 2X KO, anti-CD70 5 × 10.sup.6 1 × 10.sup.7 5 CAR+ T cells cells/mouse cells/mouse 3 3X KO (PD1), anti- 5 × 10.sup.6 1 × 10.sup.7 5 CD70 CAR+ T cells cells/mouse cells/mouse 4 3X KO (CD70,) anti- 5 × 10.sup.6 1 × 10.sup.7 5 CD70 CAR+ T cells cells/mouse cells/mouse 5 4X KO (CD70, PD1), 5 × 10.sup.6 1 × 10.sup.7 5 anti-CD70 CAR+ T cells cells/mouse cells/mouse

[0698] Tumor volume was measured 2 times weekly from day of treatment initiation. By day 5 treatment with all four types of anti-CD70 CAR T cells began to show a decrease in tumor volume and by day 22, all four types of anti-CD70 CAR T cells completely eliminated CD70+ kidney cancer tumors during the duration of the study until day 91 (FIG. **27**A). These data demonstrate that all four anti-CD70 CAR T cells can regress CD70+ kidney cancer tumors in vivo.

[0699] To test the activity of the anti-CD70 CAR T cells after rechallenge, surviving mice were inoculated in the subcutaneous left hind flank with $5×10.sup.6$ A498 renal carcinoma cells/mouse on day 25. (Table 16). Sustained efficacy was evaluated from day 46 onward. Results are shown in

FIG. **27B**. At day 56, 5 out of 5 mice treated with 2×, CD70 CAR+ T cells exhibited tumors regrowth post rechallenge, 4 out of 5 mice treated with 3×(PD1), CD70 CAR+ T cells exhibited tumors regrowth post rechallenge, 2 out of 5 mice treated with 4×(CD70, PD1), CD70 CAR+ T cells exhibited tumors regrowth post rechallenge, while none of the mice treated with 3×(CD70), CD70 CAR+ T cells exhibited tumors regrowth post rechallenge. This trend continued, at day 70, 4 out of 5 mice treated with 3×(PD1), CD70 CAR.sup.+ T cells exhibited tumors regrowth post rechallenge, 4 out of 5 mice treated with 4×(CD70, PD1), CD70 CAR.sup.+ T cells exhibited tumors regrowth post rechallenge, while only one of the mice treated with 3×(CD70), CD70 CAR.sup.+ T cells exhibited a small tumor regrowth that began to appear at 34 days post rechallenge. Even out to day 91, only 1 of 5 mice treated with 3×(CD70), CD70 CAR.sup.+ T cells was starting to exhibit tumor regrowth, indicating that TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells retain a higher in vivo efficacy after re-exposure to tumor cells.

TABLE-US-00016

TABLE 16

Size of rechallenge tumors in untreated mice or mice treated with CD70 CAR T cells

CAR T treatment	Subject	Tumor volume (mm^3) at day post CAR T cell dosing				
		Day 77	Day 81	Day 84	Day 88	Day 91
2KO, CD70 CAR T	1	1044	1265	1853	1927	2150
	2	653	927	1040	1123	1256
	3	899	1267	1603	1678	2167
	4	701	1287	1672	1817	2490
	5	689	1146	1423	1525	1901
3KO (PD1), CD70 CAR T	1	385	692	983	1172	1369
	2	0	0	0	0	0
	3	566	738	1030	1537	1740
	4	712	1111	1337	1482	1832
	5	632	778	1017	1129	1289
3KO (CD70), CD70 CAR T	1	0	0	0	0	0
	2	34	56	75	104	135
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
4KO, CD70 CAR T	1	66	91	182	215	304
	2	56	85	119	126	155
	3	0	0	0	0	0
	4	76	175	218	256	316
	5	35	30	51	58	63
No treatment	1	567	1263	1673	1751	2020
	2	882	1214	1535	1609	2047
	3	1158	1304	1676	1924	2389
	4	295	391	667	789	1078
	5	707	1213	1676	1766	2056

Treatment in Large Tumor Model

[0700] The in vivo efficacy of anti-CD70 CAR T cells against larger renal cell carcinoma tumors was investigated. As above, CRISPR/Cas9 and AAV6 were used to create human T cells that lack expression of the TCR, β2M, CD70 and/or PD1 with concomitant expression from the TRAC locus using a CAR construct targeting CD70 (SEQ ID NO: 45). In this example activated T cells were first electroporated with 2, 3 or 4 distinct Cas9:sgRNA RNP complexes containing sgRNAs targeting TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), PD1 (SEQ ID NO: 42), and CD70 (SEQ ID NO: 36 or 37). The DNA double stranded break at the TRAC locus was repaired by homology directed repair with an AAV6-delivered DNA template (SEQ ID NO: 43) (encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 5) containing right and left homology arms to the TRAC locus flanking a chimeric antigen receptor cassette (−/+ regulatory elements for gene expression).

[0701] The resulting modified T cells are 2×KO (TRAC−/β2M−), 3×KO (TRAC−/β2M−/PD1− or TRAC−/β2M−/CD70−) and 4×KO (TRAC−/β2M−/PD1−/CD70−) anti-CD70 CAR+ (with 41BB costimulatory domain) T cells. The ability of these anti-CD70 CAR+ T cells to ameliorate disease caused by a CD70+ renal carcinoma cell line was evaluated in NOG mice using methods employed by Translational Drug Development, LLC (Scottsdale, AZ). In brief, 12, 5-8 week old female mice, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. Mice received a subcutaneous inoculation of 5×10^6 A498 renal carcinoma cells/mouse. When mean tumor size reached 125-175 mm^3 (target of ~150 mm^3), the mice were further divided into 5 treatment groups as shown in Table 17. On day 1, treatment group 2 to 5 received a single 200 µl intravenous dose of anti-CD70 CAR+ T cells according to Table 17.

TABLE-US-00017

TABLE 17

Treatment groups

Group	T cell treatment CAR-T	A498 cells	(i.v)	N
1	None	5 × 10^6 cells/mouse	None	5
2	2X KO, CD70 CAR+ T cells	5 × 10^6 cells/mouse	1 × 10^7 cells/mouse	5
3	3X KO (PD1), CD70 CAR+ T cells	5 × 10^6 cells/mouse	1 × 10^7 cells/mouse	4
4	3X KO (CD70,) CD70 CAR+ T cells	5 × 10^6 cells/mouse	1 × 10^7 cells/mouse	5
5	4X KO (CD70, PD1), CD70 CAR+ T cells	5 × 10^6	1 × 10^7	5

cells/mouse cells/mouse

[0702] Tumor volume was measured 2 times weekly from day of treatment initiation. By day 4 treatment only the 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ T cells and 4×KO (TRAC−/β2M−/PD1−/CD70−) anti-CD70 CAR+ T cells began to show a decrease in tumor volume (FIG. **27**C). In contrast, the tumor growth for animals treated with 2×KO (TRAC−/β2M−) anti-CD70 CAR+ T cells or 3×KO (TRAC−/β2M−/PD1−) anti-CD70 CAR+ T cells was similar to the no treatment group. By day 23 treatment, the 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ T cells completely eliminated CD70+ kidney cancer tumors in vivo. By day 23 treatment, elimination of the tumors in response to the 4×KO (TRAC−/β2M−/PD1−/CD70−) anti-CD70 CAR+ T cells was almost complete, with 4 of 5 mice exhibiting no detectable kidney cancer tumors in vivo.

[0703] These data show that 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ T cells and 4×KO (TRAC−/β2M−/PD1−/CD70−) anti-CD70 CAR+ T cells can significantly regress large CD70+ kidney cancer tumors in vivo.

In Vivo Tumor Model for Anti-BCMA CAR in Context of PD1, CD70, and PD1 with CD70 Knock Outs.

[0704] The efficacy of TRAC−/β2M−/anti-BCMA (4-1BB co-stim) CAR+ T cells, TRAC−/β2M−/PD-1−/anti-BCMA (4-1BB co-stim), TRAC−/β2M−/CD70−/anti-BCMA (4-1BB co-stim), and TRAC−/β2M−/PD-1−/CD70−/anti-BCMA (4-1BB co-stim) CAR+ T cells against the subcutaneous MM.1S tumor xenograft model in NOG mice was evaluated. In brief, 25, 5-8 week old female, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. On day 1, 25 mice received a subcutaneous inoculation in the right flank of $5×10^6$ MM.1S cells in 50% Matrigel/mouse. When the mean tumor volume reached between 75 and 125 mm.sup.3, the mice were divided into 5 treatment groups (N=5) and dosed with T cell populations comprising ~50% anti-BCMA CAR.sup.+ T cells, as indicated in Table 18.

TABLE-US-00018 TABLE 18 Dosing # of T Cells Anti-BCMA Group CAR T Cell injected CAR+ T cells N 1 N/A N/A N/A 5 2 TRAC-/β2M-/ 1 × 10.sup.7 5 × 10.sup.6 5 anti-BCMA cells/mouse (5 million) 3 TRAC-/β2M-/ 1 × 10.sup.7 5 × 10.sup.6 5 PD-1-/anti-BCMA cells/mouse (5 million) 4 TRAC-/β2M-/ 1 × 10.sup.7 5 × 10.sup.6 5 CD70-/anti-BCMA cells/mouse (5 million) 5 TRAC-/β2M-/ 1 × 10.sup.7 5 × 10.sup.6 5 PD-1-/CD70-/ cells/mouse (5 million) anti-BCMA

[0705] Tumor volume and body weights were measured twice weekly and individual mice were euthanized when their tumor volume reached ≥2000 mm.sup.3.

[0706] By day 16, all treatment groups showed tumor regression from the starting volumes while animals in the control group had tumors averaging greater than 1500 mm.sup.3. By day 27, all animals in the control group had reached the tumor volume endpoint of ≥2000 mm.sup.3 while all treatment groups had an average tumor volume less than 20 mm.sup.3 (FIG. **28**A). On day 45, all mice from each treatment group (Groups 2-5) were further subjected to a secondary tumor challenge (re-challenge). The mice received a second subcutaneous inoculation in the left flank of $5×10^6$ MM.1S cells in 50% Matrigel/mouse. A new group of control mice were entered (N=5) and also received an inoculation of $5×10^6$ MM.1S cells in 50% Matrigel/mouse in the left flank.

[0707] All mice were monitored for tumor growth in both the initial right flank tumor and the rechallenge tumor in the left flank. All treatment groups successfully inhibited tumor growth in the initial right flank tumor in most subjects (FIG. **28**A; Table 19). Tumor growth was inhibited by all treatments both before and after tumor re-challenge for the duration of the experiment to day 77. Only one subject treated with TRAC−/β2M−/CD70−/PD1−/anti-BCMA CAR.sup.+ T cells exhibited tumor growth from the initial cancer cell challenge (Table 19).

[0708] Surprisingly, tumor growth after re-challenge in the left flank was also significantly inhibited by all treatment groups from the date of re-challenge (day 45) to day 77 (FIG. **28**B; Table 19). These data demonstrate that the CAR+ T cells persist in vivo to inhibit initial tumor growth, as

well as inhibiting growth of new tumors following a re-challenge with additional cancer cells even though no further CAR-T cells were delivered to these mice. For example, three of the four mice initially treated with populations of TRAC−/β2M−/anti-BCMA CAR.sup.+ T cells, three of the five mice initially treated with TRAC−/β2M−/CD70−/anti-BCMA CAR.sup.+ T cells, and three of the five mice initially treated with TRAC−/β2M−/PD1−/anti-BCMA CAR.sup.+ T cells, exhibited no new tumor growth despite a second challenge (re-challenge) to new cancer cells. These data demonstrate that, unexpectedly, anti-BCMA CARP T cells are capable of persisting for long periods of time in vivo, e.g., up to at least 77 days following injection, and retain their ability to inhibit tumor cell growth and reduce tumor volumes for long periods in vivo. These surprising results indicate that use of such TRAC−/β2M−/anti-BCMA CAR.sup.+ T cells would achieve superior long-term anti-cancer effect in vivo.

[0709] Human CD45+ cells were quantified from mouse blood using BD Trucount tubes following the manufacturers protocol and detected using Brilliant Violet 786 conjugated anti-human CD45 (Biolegend Cat #368528). All groups showed values of less than 100 huCD45+ cells/μl at 1 week. Two weeks post dosing, the number of circulating CD45+ in all groups peaked before falling to pre-week 1 values by week 3 (FIG. **29**). Upon re-challenge at Day 45, the anti-BCMA CAR+ T cell treated subjects were able to eliminate or inhibit tumor growth without subsequent expansion of circulating CAR T cells following cancer rechallenge. These data further demonstrate that, unexpectedly, anti-BCMA CAR+ T cells are capable of persisting for long periods of time in vivo, e.g., up to at least 77 days following injection, and retain their ability to inhibit tumor cell growth and reduce tumor volumes for long periods in vivo. These surprising results indicate that use of TRAC−/β2M−/anti-BCMA CAR+ T cells, TRAC−/β2M−/CD70−/anti-BCMA CAR+ T cells and TRAC−/β2M−/PD1−/anti-BCMA CAR+ T cells would achieve superior long-term anti-cancer effect in vivo.

TABLE-US-00019 TABLE 19 Size of tumors in untreated mice or mice treated with anti-BCMA CAR T cells Tumor volume Tumor volume at Day 45 (mm.sup.3) at Day 77 (mm.sup.3) Treatment Mouse Right Flank Right Flank Left Flank No Treatment 1 TS TS TS 2 TS TS TS 3 TS TS TS 4 TS TS TS 5 TS TS TS TRAC-/β2M-/ 1 0 0 (MS at 0 (MS at anti-BCMA Day 59) Day 59:) 2 0 0 0 3 0 0 0 4 0 0 297 5 FD-T at FD-T at FD-T at day 16 day 16 day 16 TRAC-/ 1 0 0 1820 β2M-/PD1-/ 2 0 0 0 anti-BCMA 3 0 0 487 4 0 0 0 5 0 0 0 TRAC-/ 1 10 0 0 β2M-CD70-/ 2 0 0 0 anti-BCMA 3 0 0 (TS at 2349 (TS at day 77) day 77) 4 0 0 0 5 0 0 258 TRAC-/β2M-/ 1 0 0 1157 PD1-/CD70-/ 2 0 0 1842 anti-BCMA 3 0 0 1664 4 0 0 0 5 89 1583 (TS at 1560 (TS at day 73) day 73) TS = sacrificed because of tumor volume; MS = Moribund sacrifice; FD-T = animal found dead.

In Vivo Tumor Model for Anti-BCMA CAR in Context of CD70 Knockout: Effect of CD70 KO on Moderate CAR T Dosing

[0710] The efficacy of several anti-BCMA CAR.sup.+ T cell genotypes, both with and without CD70 knockouts, was evaluated against the subcutaneous RPMI-8226 tumor xenograft model in NOG mice. In brief, eighty five (85), 5-8 week old female, CIBA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. On day 1 mice received a subcutaneous inoculation of 10×10.sup.6 RPMI-8226 cells/mouse. Ten (10) days post inoculation with RPMI-8226 cells, the mice were divided into 17 treatment groups (N=5) and dosed with cell populations comprising ˜80% anti-BCMA CAR m cells, as indicated in Table 20.

TABLE-US-00020 TABLE 20 Anti-BCMA # of T Cells Anti-BCMA Group CAR T Cell injected CAR+ T cells N 1 N/A N/A N/A 5 2 TRAC-/β2M-/ 3 × 10.sup.6 2.4 × 10.sup.6 5 anti-BCMA cells/mouse (2.4 million) 3 TRAC-/β2M-/ 1 × 10.sup.6 .sup. 8 × 10.sup.5 5 anti-BCMA cells/mouse (0.8 million) 4 TRAC-/β2M-/ 3 × 10.sup.5 2.4 × 10.sup.5 5 anti-BCMA cells/mouse (0.24 million) 5 TRAC-/β2M-/ 1 × 10.sup.5 .sup. 8 × 10.sup.4 5 anti-BCMA cells/mouse (0.08 million) 6 TRAC-/β2M-/ 3 × 10.sup.6 2.4 × 10.sup.6 5 PD1-/anti-BCMA cells/mouse (2.4 million) 7 TRAC-/β2M-/ 1 × 10.sup.6 .sup. 8 × 10.sup.5 5 PD1-/anti-BCMA cells/mouse (0.8

million) 8 TRAC-/β2M-/ 3 × 10.sup.5 2.4 × 10.sup.5 5 PD1-/anti-BCMA cells/mouse (0.24 million) 9 TRAC-/β2M-/ 1 × 10.sup.5 .sup.   8 × 10.sup.4 5 PD1-/anti-BCMA cells/mouse (0.08 million) 10 TRAC-/β2M-/ 3 × 10.sup.6 2.4 × 10.sup.6 5 CD70-/anti-BCMA cells/mouse (2.4 million) 11 TRAC-/β2M-/ 1 × 10.sup.6 .sup.   8 × 10.sup.5 5 CD70-/anti-BCMA cells/mouse (0.8 million) 12 TRAC-/β2M-/ 3 × 10.sup.5 2.4 × 10.sup.5 5 CD70-/anti-BCMA cells/mouse (0.24 million) 13 TRAC-/β2M-/ 1 × 10.sup.5 .sup.   8 × 10.sup.4 5 CD70-/anti-BCMA cells/mouse (0.08 million) 14 TRAC-/β2M-/ 3 × 10.sup.6 2.4 × 10.sup.6 5 PD1-/CD70-/ cells/mouse (2.4 million) anti-BCMA 15 TRAC-/β2M-/ 1 × 10.sup.6 .sup.   8 × 10.sup.5 5 PD1-/CD70-/ cells/mouse (0.8 million) anti-BCMA 16 TRAC-/β2M-/ 3 × 10.sup.5 2.4 × 10.sup.5 5 PD1-/CD70-/ cells/mouse (0.24 million) anti-BCMA 17 TRAC-/β2M-/ 1 × 10.sup.5 .sup.   8 × 10.sup.4 5 PD1-/CD70-/ cells/mouse (0.08 million)   anti-BCMA

[0711] Tumor volume and body weight was measured twice weekly, and individual mice were euthanized when tumor volume was ≥2000 mm.sup.3. By day 22, the data show a statistically significant decrease in the tumor volume in response to higher doses of anti-BCMA CAR T cells (1×10.sup.5-3×10.sup.6 cell doses) compared to any anti-BCMA CAR T cell genotype dosed at 100,000 cells (groups 5, 9, 13 and 17) (FIG. **30**; Table 21).

[0712] At day 36, the TRAC−/β2M−/CD70−/anti-BCMA CAR+ T cells dosed at a moderate dose of 3×10.sup.5 cells exhibited a greater effect on decreasing tumor volume than the anti-BCMA CAR+ T cells without a CD70 KG (e.g., TRAC−/β2M−/anti-BCMA CAR+ T cells, TRAC−/β2M−/PD1−/anti-BCMA CAR+ T cells, or TRAC−/β2M−/PD1−/CD70−/anti-BCMA CAR+ T cells) (FIG. **30**). All of the higher doses of 1×10.sup.6 or greater all anti-BCMA CAR+ T cells (FIG. **30**; 1 Mil, 3 Mil), showed complete regression in tumor volume. This trend continued out to Day 57 of the study.

[0713] These results demonstrate that inhibiting the activity of CD70 (e.g., by knocking out CD70) increases the efficacy and potency of CAR.sup.+ T cells in vivo. This effect is independent of the presence of an anti-CD70 CAR.

TABLE-US-00021 TABLE 21 Anti-BCMA CAR+ Tumor Volume (mm.sup.3) Tumor Volume (mm.sup.3) Group Treatment T cells/dose at Day 36 at Day 57 1 No N/A 220 220 186 173 278 1790 1794 938 2055 2029 Treatment 2 TRAC-/β2M-/ 2.4 × 10.sup.6 0 0 0 0 0 0 0 0 0 0 anti- BCMA (2.4 million) 3 .sup.   8 × 10.sup.5 0 0 0 0 0 0 0 0 0 0 (0.8 million) 4 2.4 × 10.sup.5 65 65 77 56 0 516 441 257 97 337 (0.24 million)   5 .sup.   8 × 10.sup.4 264 264 386 276 185 2391 2283 2058 2147 2359 (0.08 million)   6 TRAC-/β2M-/ 2.4 × 10.sup.6 0 0 0 0 0 0 0 0 0 0 PD1-/anti- (2.4 million) 7 BCMA .sup.   8 × 10.sup.5 0 0 0 0 0 0 0 0 0 0 (0.8 million) 8 2.4 × 10.sup.5 135 135 59 57 28 764 518 280 181 79 (0.24 million)   9 .sup.   8 × 10.sup.4 261 261 265 287 312 1532 1557 2218 2010 2098 (0.08 million)   10 TRAC-/β2M-/ 2.4 × 10.sup.6 0 0 0 0 0 0 0 0 0 0 CD70-/anti- (2.4 million) 11 BCMA .sup.   8 × 10.sup.5 0 0 0 0 0 0 0 0 0 0 (0.8 million) 12 2.4 × 10.sup.5 47 47 0 0 0 526 58 47 0 0 (0.24 million)   13 .sup.   8 × 10.sup.4 292 292 267 313 235 2075 2127 2096 1365 2354 (0.08 million)   14 TRAC-/β2M-/ 2.4 × 10.sup.6 0 0 0 0 0 0 0 0 0 0 PD1-/CD70-/ (2.4 million) 15 anti-BCMA .sup.   8 × 10.sup.5 0 0 0 0 0 0 0 0 0 0 (0.8 million) 16 2.4 × 10.sup.5 100 100 91 19 20 478 576 82 131 289 (0.24 million)   17 .sup.   8 × 10.sup.4 310 310 319 345 451 1528 2160 2843 2557 1499 (0.08 million)

Example 9. Multi Knockout CAR T Cells Retain Cytokine Dependency

[0714] Cytokine Dependency. To determine whether gene editing resulted in unwanted off-target editing that could generate cells with adverse properties, such as uncontrolled cell growth, the ability gene edited CAR T cells to grow in the absence of cytokines and/or serum was assessed.

[0715] Anti-CD70 CAR T cells: The ability of TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ cells to grow in the absence of cytokines and/or serum was assessed. 5×10.sup.6 TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ cells were plated ~2 weeks post cell production (Day 0). The number of viable cells were enumerated 7 and 14 days post plating in either full media, 5% human serum without cytokines (IL-2 and IL-7), or base media lacking serum

and cytokines. No cells were detected at 14 days plated in the cultures that lacked cytokines, suggesting that any potential off-target effects due to genome editing did not induce growth factor independent growth/proliferation to the cells (FIG. **31**). The cells only proliferated in the presence of cytokines (full media that contains cytokines) and did not proliferate in the presence of serum alone. Thus, in vivo, the cells would likely not grow in the absence of cytokine, growth factor or antigen stimulation due to any off-target genome editing.

[0716] The ability of TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD1.sup.− anti-CD70 CAR.sup.+ cells to grow in the absence of cytokines and/or serum was also assessed. 2×10.sup.6 cells were plated ˜2 weeks post cell production (Day 0). The number of viable cells were enumerated until 26 days post plating in either full media, 5% human serum without cytokines (IL-2 and IL-7), or base media lacking serum and cytokines. No cells were detected at 26 days plated in the cultures that lacked cytokines, suggesting that any potential off-target effects due to genome editing did not induce growth factor independent growth/proliferation to the cells (FIG. **32**). The cells only proliferated in the presence of cytokines (full media that contains cytokines) and did not proliferate in the presence of serum alone. Thus, genome editing did not induce any adverse events that allow the cells to grow in the absence of cytokine, growth factor or antigen stimulation.

[0717] Anti-BCMA CAR+ T cells: The ability of TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD-1.sup. −/anti-BCMA CAR.sup.+ cells to grow in the absence of cytokines and/or serum was assessed. TRAC.sup.−/β2M.sup.−/CD70.sup.−/PD-1.sup.−/anti-BCMA CAR.sup.+ cells are also referred to as 4×KO, BCMA CAR.sup.+ cells. 1×10.sup.6 4×KO, BCMA CAR+ cells were plated following the 10 rechallenges described in Example 6. The number of viable cells were enumerated 7 and 14 days post plating in either full media, 5% human serum without cytokines (IL-2 and IL-7), or base media lacking serum and cytokines. No cells were detected at 13 days plated in the cultures that lacked cytokines, suggesting that any potential off-target effects due to genome editing did not induce growth factor independent growth/proliferation to the cells (FIG. **33**). The cells only proliferated in the presence of cytokines (full media that contains cytokines) and did not proliferate in the presence of serum alone. Thus, in vivo, the cells would likely not grow in an uncontrolled way.

[0718] Other CAR T cells: It has previously been shown that the anti-CD33 CAR+ T cells and anti-CD19 CAR+ T cells exemplified herein only proliferated in the presence of cytokines and do not proliferate in the presence of serum alone. Thus, in vivo, these cells would likely not grow in an uncontrolled way.

[0719] Cytokine Release Assay. To measure cytokine release, T cells and target cells were co-incubated for 24 hours at the ratios indicated. Supernatant media was collected for use in IL-2 or IFNγ ELISAs (RD Systems) on a new plate following the manufacturer's instructions (RD Systems).

[0720] The ability of the TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ cells to produce interleukin-2 (IL-2) when co-cultured in the presence of A498 cells was analyzed using the ELISA assay. Both the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells secreted high levels of IL-2. Strikingly, the TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ cells secreted higher levels of IL-2 than the TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ cells when cultured with A498 cells (FIG. **34**). These results suggest that knocking-out the CD70 gene gives an advantage to anti-CD70 CAR.sup.+ T cells to secrete more IL-2.

Example 10. Effect of Multiple Knockout on Anti-CD70 CAR+ T Cells on A498 Renal Carcinoma Cells

Effect of Multi Knock-Out on the Function of Anti-CD70 CAR+ T Cells.

[0721] Cell Killing Assay. The ability of multi-gene editing to kill A498 renal carcinoma cells was determined using the cell kill assay described above. In brief, the TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ (2×KO, CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70

CAR.sup.+ (3×KO (PD-1), CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (3×KO (CD70), CD70 CAR.sup.+) and TRAC.sup.−/β2M.sup.−/PD-1.sup. −/CD70.sup.−/anti-CD70 CAR.sup.+ (4×KO, CD70 CAR.sup.+) cells were incubated with a CD70.sup.+ adherent RCC-derived cell line (A498 cells) at various CAR T cell:A498 target cells ratios.

[0722] The TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ cells exhibited potent cell killing of RCC-derived cells following 24-hour co-incubation (FIG. **35**). The quadruple TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T demonstrated higher cell kill potency than triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells that demonstrated higher potency than double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells (visible at low T-cell: A498 Ratio of 0.5:1 and 0.25:1). The results demonstrate knocking out both the CD70 and PD-1 genes gave the anti-CD70 CAR+ cells higher cell kill potency.

[0723] The gene edited cells also exhibited potent cell killing of RCC-derived cells following 24-hour co-incubation at a CAR T cell:A948 target cell ratio of 0.24:1. (FIG. **36**). Specifically, the triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and the quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells demonstrated higher potency than the double knockout TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ T cells or the triple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ T cells. These data indicate that knockout of CD70 in the context of an anti-CD70 CAR improved the cell killing ability of the anti-CD70 CAR.sup.+ T cells.

[0724] Cytokine Release Assay. A cytokine release assay was performed as described above. The ability of the double knockout, triple knockout, and quadruple knockout anti-CD70 CAR.sup.+ T cells to produce IL-2 and interferon gamma (IFN-gamma (IFN-g)) when co-cultured in the presence of A498 cells following 24-hour co-incubation at a ratio (CAR T cell:A498 target cell) of 0.25:1 was assessed using an ELISA assay. IL-2 and IFN-g from supernatants of cell co-cultures were measured. The triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells secreted the highest levels of IFN-g (FIG. **37**A) and IL-2 (FIG. **37**B) when cultured with A498 cells.

Effect of CD70 Knockout on Exhaustion Marker Expression

[0725] The levels of the exhaustion markers PD-1 and LAG3 were assessed on the TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ (2×KO, CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ (3×KO (PD-1), CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (3×KO (CD70), CD70 CAR.sup.+) and TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (4×KO, CD70 CAR.sup.+) T cells used in the Examples above. CD4.sup.+ T cells were assessed for PD-1 expression (FIG. **38**) and both CD8.sup.+ T cells and CD4.sup.+ T cells were assessed for LAG3 expression (FIG. **39**A and FIG. **39**B, respectively) by flow cytometry.

[0726] The data demonstrate that CD70 KO reduces exhaustion marker expression in CAR T cells. The data in FIG. **38** shows that PD-1 expression is decreased, as expected, when PD-1 is knocked out, and it is also decreased when CD70 is knocked out.

[0727] The data in FIGS. **39**A and **39**B show that knocking out CD70, reduces the LAG3 expression marker in CD4 and CD8 cells.

[0728] The data demonstrate that knocking out CD70, specifically, could reduce the potential exhaustion of the CD8.sup.+ and CD4.sup.+ gene edited populations of CAR+ T cells leading to better therapeutics.

Example 11. CD70 KO Improves Cell Kill in Multiple Cell Types

[0729] CD70 Expression in Various Cancer Cell Lines. Relative CD70 expression was measured in various cancer cell lines to further evaluate the ability of anti-CD70 CAR.sup.+ T cells to kill

various cancer types. CD70 expression was measured by FACS analysis using Alexa Fluor 647 anti-human CD70 antibody (BioLegend Cat. No. 355115). FIG. **40**A (left graph) shows the relative expression of CD70 in ACHN cells, as measured by FACS, compared to other kidney cancer cell lines A498, 786-O, cacki-1 and Caki-2. Additionally, non-kidney cancer cell lines were evaluated for CD70 expression by FACS analysis (Table 22, FIG. **40**A and FIG. **40**B) using either an Alexa Fluor 647 anti-human CD70 antibody (BioLegend Cat. No. 355115; FIG. **40**A, right panel) or a FITC anti-human CD70 antibody (BioLegend Cat. No. 355105) in FIG. **40**B. SNU-1 (intestinal cancer cells) exhibited high levels of CD70 expression that were similar to A498 (FIG. **40**A, right panel). SKOV-3 (ovarian), HuT78 (lymphoma), NCI-H1975 (lung) and Hs-766T (pancreatic) cell lines exhibited levels of CD70 expression that were similar or higher than ACHN but lower than A498 (Table 22, FIG. **40**B).

TABLE-US-00022 TABLE 22 Relative CD70 Cell Line Cancer type expression A498 Kidney Carcinoma High ACHN Kidney (derived from Medium-Low metastasis) SK-OV-3 Ovarian Adenocarcinoma Medium NCI-H1975 Lung Adenocarcinoma (NSCLC) Medium Calu-1 Lung Carcinoma Low DU 145 Prostate Carcinoma Low SNU-1 Gastric Carcinoma High Hs 766T Pancreatic Carcinoma Medium MJ T cell Lymphoma High HuT78 T cell Lymphoma Medium HuT102 T cell Lymphoma Medium PANC-1 Pancreatic Carcinoma Low U937 AML No expression K562 chronic myelogenous leukemia No expression (Negative Control)

[0730] Cell Kill Assay. The ability of multi-gene edited anti-CD70 CAR+ cells to kill ACHN renal carcinoma cells was determined using the cell kill assay described above. The TRAC.sup.−/β2M.sup.−/anti-CD70 CAR.sup.+ (2×KO, CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/PD-1.sup.−/anti-CD70 CAR.sup.+ (3×KO (PD-1), CD70 CAR.sup.+), TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (3×KO (CD70), CD70 CAR.sup.+) and TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (4×KO, CD70 CAR.sup.+) cells were incubated with an adherent RCC-derived cell line expressing low levels of CD70 antigen (ACHN cells) (FIG. **40**A shows the relative expression of CD70 in ACHN cells, as measured by FACS, compared to other kidney cancer cell lines A498, 786-O, cacki-1 and Caki-2) at a CAR T cell:ACHN target cells ratio of 0.5:1 (FIG. **40**C) and 0.25:1 (FIG. **40**D). The gene edited cells exhibited potent cell killing of RCC-derived cells following 24-hour co-incubation (FIGS. **40**C and **40**D). The cells demonstrated higher potency when PD-1 was knocked out, when CD70 was knocked out, and even slightly higher potency when both PD-1 and CD70 were knocked out. In conclusion, knockout of PD-1 or CD70 or of both PD-1 and CD70 together improves the cell killing ability of the anti-CD70 CAR+ cells in ACHN cells.

[0731] Although ACHN cells were found to express moderate to low levels of CD70, they were surprisingly susceptible to killing by 3×KO (PD-1), CD70 CAR+ T cells, 3×KO (CD70), CD70 CAR+ T cells, and 4×KO CD70 CAR+ T cells (FIGS. **40**C and **40**D). This indicates that high CD70 expression is not a requirement for effective killing of a target cell by gene-edited T cells that express an anti-CD70 CAR. Additionally, given that the levels of CD70 expression on SNU-1, SK-OV-3, NCI-H1975 and HS-766T cell lines were found to be similar or higher than ACHN, it was expected that anti-CD70 CAR.sup.+ T cells would be especially efficient at killing these cancer cell types as well. Indeed, it was found that TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (4×KO, CD70 CAR.sup.+) and TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ (3×KO (CD70), CD70 CAR.sup.−) exhibited surprisingly potent cell killing of numerous solid tumor cell lines after only 24 hours of co-culture (FIG. **40**E shows killing by 4×KO CAR+ T cells and FIG. **40**F shows killing by 3×KO CAR+ T cells). Both 3×KO, CD70 CAR+ and 4×KO, CD70 CAR+ T cells killed >60% of kidney, pancreatic, and ovarian tumor cells (A498, ACHN, SK-OV-3, and Hs-766T) at a 4:1 effector:target cell ratio and >50% at a 1:1 effector:target cell ratio. Cell killing of cancer cell lines that had medium to low CD70 expression (NCI-H1975, Calu-1 and DU 145) was still effective with >30% killing at an effector:target cell ratio of 4:1 within 24 hours of co-culture (FIGS. **40**E and **40**F). Longer exposure (i.e., 96 hours) to either

3×KO or 4×KO, CD70 CAR+ T cells resulted in an increase in cancer cell killing across all cell types, particularly for SKOV-3, Hs-766T, and NIC-H1975 cells wherein killing was >80% at an effector:target cell ratio of 1:1 (FIG. **40**G).

SNU-1 Cell Kill by was Assessed by Visual Assessment.

[0732] Target cell killing following long exposure to CAR+ T cells was also assessed by microscopy for SNU-1 cancer cells. SNU-1 cells were plated at a density of 1 million cells per well in a 6 well plate and mixed at an effector:target ratio of 4:1 with 3×KO (CD70), anti-CD70 CAR.sup.+ T cells. The co-culture was incubated for six (6) days and the presence of viable cancer cells was assessed by microscope. All gastric carcinoma target cells (SNU-1) were eliminated in wells containing TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells, as compared to control wells, indicating cancer cells were completely eliminated by anti-CD70 CAR.sup.+ T cells with an extended co-culture.

[0733] The ability of anti-CD70 CAR+ T cells to selectively kill CD70-expressing cells was determined. A flow cytometry assay was designed to test killing of cancer cell suspension lines (e.g., K562, MM.1S and HuT78 cancer cells that are referred to as "target cells") by 3×KO (CD70) (TRAC.sup.−/B2M.sup.−/CD70.sup.−) anti-CD70 CAR+ T cells. Two of the target cell lines that were used were CD70-expressing cancer cells (e.g., MM.1S and HuT78), while a third that was used as negative control cancer cells lack CD70 expression (e.g., K562). The TRAC.sup. −/B2M-/CD70-/anti-CD70 CAR+ T cells were co-cultured with either the CD70-expressing MM.1S or HuT78 cell lines or the CD70-negative K562 cell line. The target cells were labeled with 5 μM efluor670 (eBiosciences), washed and seeded at a density of 50,000 target cells per well in a 96-well U-bottom plate. The target cells were co-cultured with TRAC.sup.−/B2M.sup.−/CD70.sup. − anti-CD70 CAR+ T cells at varying ratios (0.5:1, 1:1, 2:1 and 4:1 CAR+ T cells to target cells) and incubated overnight. Target cell killing was determined following a 24 hour co-culture. The cells were washed and 200 μL of media containing a 1:500 dilution of 5 mg/mL DAPI (Molecular Probes) (to enumerate dead/dying cells) was added to each well. Cells were then analyzed by flow cytometry and the amount of remaining live target cells was quantified.

[0734] FIG. **40**H, FIG. **40**I, and FIG. **40**J demonstrate selective target cell killing by TRAC−/B2M−/CD70− anti-CD70 CAR+ T cells. A 24 hour co-culture with 3×KO (CD70) CAR+ T cells resulted in nearly complete killing of T cell lymphoma cells (HuT78), even at a low CAR+ T cell to CD70-expressing target cell ratio of 0.5:1 (FIG. **40**J). Likewise, a 24 hour co-culture resulted in nearly complete killing of multiple myeloma cells (MM.1S) at all CAR+ T cell to target cell ratios tested (FIG. **40**I). Killing of target cells was found to be selective in that TRAC−/B2M−/anti-CD70 CAR+ T cells induced no killing of CD70-deficient K562 cells that was above the level of control samples (e.g., either cancer cells alone or co-culture with no RNP T cells) at any effector:target cell ratio tested (FIG. **40**H).

[0735] Cytokine Release Assay. A cytokine release assay was performed as described above. The ability of the double knockout, triple knockout, and quadruple knockout anti-CD70 CAR.sup.+ T cells to produce IL-2 and IFN-g when co-cultured in the presence of ACHN cells following 24-hour co-incubation at a ratio (CAR T cell:ACHN target cell) of 0.25:1 was assessed using an ELISA assay. IL-2 and IFN-g from supernatants of cell co-cultures were measured. The triple knockout TRAC.sup.−/β2M.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells and quadruple knockout TRAC.sup.−/β2M.sup.−/PD-1.sup.−/CD70.sup.−/anti-CD70 CAR.sup.+ T cells secreted the highest levels of IFN-g (FIG. **41**) and IL-2 (FIG. **41**B) when cultured with ACHN cells. In conclusion, knockout of CD70 or of both PD-1 and CD70 together improves the cell killing ability of the anti-CD70 CAR+ cells in ACHN cells.

Example 12. Efficacy of CD70 KO in Anti-CD70 CAR+ T Cells: The Tumor Xenograft Model in NOG Mice

Treatment in the Ovarian Tumor Model

[0736] The ability of T cells expressing an anti-CD70 CAR to eliminate ovarian adenocarcinoma

cells that express moderate levels of CD70 was evaluated in vivo using a subcutaneous ovarian carcinoma (SKOV-3) tumor xenograft model in mice.

[0737] CRISPR/Cas9 and AAV6 were used as above (see for example, Example 3) to generate human T cells that lack expression of the TCR, β2M, CD70 with concomitant expression from the TRAC locus using a CAR construct targeting CD70 (SEQ ID NO: 45; SEQ ID NO: 46. In this example activated T cells were first electroporated with 3 distinct Cas9:sgRNA RNP complexes containing sgRNAs targeting TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), and CD70 (SEQ ID NO: 36 or 37). The DNA double stranded break at the TRAC locus was repaired by homology directed repair with an AAV6-delivered DNA template comprising a donor template (SEQ ID NO: 44; SEQ ID NO: 45) (encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 45) containing right and left homology arms to the TRAC locus flanking a chimeric antigen receptor cassette (−/+ regulatory elements for gene expression).

[0738] The resulting modified T cells are 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ T cells. The ability of these anti-CD70 CAR+ T cells to ameliorate disease caused by a CD70+ ovarian carcinoma cell line was evaluated in NOG mice using methods employed by Translational Drug Development, LLC (Scottsdale, AZ). In brief, 12 5-8 week old female, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. Mice received a subcutaneous inoculation of 5×10.sup.6 SKOV-3 ovarian carcinoma cells/mouse in the right hind flank. When mean tumor size reached 25-75 mm.sup.3 (target of ~50 mm.sup.3), the mice were further divided into two treatment groups as shown in Table 23. On Day 1, treatment group 2 received a single 200 μl intravenous dose of anti-CD70CAR+ T cells according to Table 23.

TABLE-US-00023 TABLE 23 Treatment groups T cell SKOV-3 treatment Group CAR-T cells (i.v.) N 1 None 5 × 10.sup.6 None 5 cells/mouse 2 3X KO (CD70,) 5 × 10.sup.6 1 × 10.sup.7 5 anti-CD70 CAR+ T cells cells/mouse cells/mouse

[0739] Tumor volume was measured 2 times weekly from day of treatment initiation. By day 9 post-injection, tumors treated with anti-CD70 CART cells began to show a decrease in tumor volume relative to tumors in untreated animals. By day 17 post-injection, CD70+ ovarian cancer tumors in mice treated with anti-CD70 CAR T cells were completely eliminated. This complete regression of tumor growth was sustained in treated animals through day 44 post-injection, whereupon 4 out of 5 mice treated with anti-CD70 CART cells remained tumor-free until the end-of-observation (day 69) (FIG. **42**A). These data demonstrate that 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ cells are highly potent in vivo for treating human ovarian tumors.

Treatment in the Non-Small Cell Lung Carcinoma (NSCLC) Tumor Model

[0740] The ability of T cells expressing a CD70 CAR to eliminate lung adenocarcionma cells that express moderate levels of CD70 was evaluated in in vivo using a subcutaneous lung carcinoma (NCI-H1975) tumor xenograft model in mice.

[0741] CRISPR/Cas9 and AAV6 were used as above (see for example, Example 3) to create human T cells that lack expression of the TCR, β2M, CD70 with concomitant expression from the TRAC locus using a CAR construct targeting CD70 (SEQ ID NO: 43; SEQ ID NO: 44). In this example activated T cells were first electroporated with 3 distinct Cas9:sgRNA RNP complexes containing sgRNAs targeting TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), and CD70 (SEQ ID NO: 36 or 37). The DNA double stranded break at the TRAC locus was repaired by homology directed repair with an AAV6-delivered DNA template (SEQ ID NO: 43; SEQ ID NO: 44) (encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 45) containing right and left homology arms to the TRAC locus flanking a chimeric antigen receptor cassette (−/+ regulatory elements for gene expression).

[0742] The resulting modified T cells are 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ (with 41BB costimulatory domain) T cells. The ability of these anti-CD70 CAR+ T cells to ameliorate

disease caused by a CD70+ lung carcinoma cell line was evaluated in NOG mice using methods employed by Translational Drug Development, LLC (Scottsdale, AZ). In brief, 12, 5-8 week old female, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. Mice received a subcutaneous inoculation of 5×10^6 NCI-H1975 lung carcinoma cells/mouse in the right hind flank. When mean tumor size reached 25-75 mm.sup.3 (target of ˜50 mm.sup.3), the mice were further divided into 2 treatment groups as shown in Table 24. On Day 1, treatment group 2 received a single 200 μl intravenous dose of anti-CD70CAR+ T cells according to Table 24.

TABLE-US-00024 TABLE 24 Treatment groups T cell NCI-H1975 treatment Group CAR-T cells (i.v.) N 1 None 5 × 10.sup.6 None 5 cells/mouse 2 3X KO (CD70,) 5 × 10.sup.6 1 × 10.sup.7 5 anti-CD70 CAR+ T cells cells/mouse cells/mouse

[0743] Tumor volume was measured 2 times weekly from day of treatment initiation. By day 12 post-injection, tumors treated with anti-CD70 CAR T cells began to show a decrease in tumor volume relative to tumors in untreated animals. This complete regression of tumors in treated animals continue through day 33 post injection. Treatment with anti-CD70 CAR T cells resulted in potent activity against established H1975 lung cancer xenografts through 40 days post injection (tumor regrowth was suppressed in all mice up to day 40 with tumor size <100 mm.sup.3), whereupon tumors began to grow. (FIG. **42**B). These data demonstrate that 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ cells have potent activity against human CD70+ lung cancer tumors in vivo.

Treatment in the Pancreatic Tumor Model

[0744] The ability of T cells expressing a CD70 CAR to eliminate pancreatic carcinoma cells that express moderate levels of CD70 was evaluated in in vivo using a subcutaneous pancreatic (Hs 766T) tumor xenograft model in mice.

[0745] CRISPR/Cas9 and AAV6 were used as above (see for example, Example 3) to create human T cells that lack expression of the TCR, β2M, CD70 with concomitant expression from the TRAC locus using a CAR construct targeting CD70 (SEQ ID NO: 43; SEQ ID NO: 44). In this example activated T cells were first electroporated with 3 distinct Cas9:sgRNA RNP complexes containing sgRNAs targeting TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), and CD70 (SEQ ID NO: 36 or 37). The DNA double stranded break at the TRAC locus was repaired by homology directed repair with an AAV6-delivered DNA template (SEQ ID NO: 43; SEQ ID NO: 44) (encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 45) containing right and left homology arms to the TRAC locus flanking a chimeric antigen receptor cassette (−/+ regulatory elements for gene expression).

[0746] The resulting modified T cells are 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ T cells. The ability of these anti-CD70 CAR+ T cells to ameliorate disease caused by a CD70+ pancreatic carcinoma cell line was evaluated in NOG mice using methods employed by Translational Drug Development, LLC (Scottsdale, AZ). In brief, 12, 5-8 week old female, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. Mice received a subcutaneous inoculation of 5×10^6 Hs766T pancreatic carcinoma cells in the right hind flank. When mean tumor size reached 25-75 mm.sup.3 (target of ˜50 mm.sup.3), the mice were further divided into 2 treatment groups as shown in Table 25. On Day 1, treatment group 2 received a single 200 μl intravenous dose of anti-CD70 CAR+ T cells according to Table 25.

TABLE-US-00025 TABLE 25 Treatment groups T cell Hs766T treatment Group CAR-T cells (i.v.) N 1 None 5 × 10.sup.6 None 5 cells/mouse 2 3X KO (CD70,) 5 × 10.sup.6 1 × 10.sup.7 5 anti-CD70 CAR+ T cells cells/mouse cells/mouse

[0747] Tumor volume was measured 2 times weekly from day of treatment initiation. By Day 15 post-injection, tumors treated with anti-CD70 CAR T cells began to show a decrease in tumor

volume in all treated mice. Treatment with anti-CD70 CAR+ T cells effectively reduced the size of the CD70+ pancreatic cancer tumors, in all mice tested (<37 mm.sup.3) with no evidence of further growth for the duration of the study (through Day 67) (FIG. **42**C). These data demonstrate that 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ cells induce regression of human CD70+ pancreatic cancer tumors in vivo, with potent activity against established Hs766T pancreatic cancer xenografts and durable responses beyond 60 days following treatment initiation.

Treatment in the Cutaneous T-Cell Lymphoma Tumor Xenograft Model

[0748] The ability of T cells expressing an anti-CD70 CAR to eliminate T cell lymphoma was evaluated in in vivo using a subcutaneous T-cell lymphoma (Hu T78) tumor xenograft model in mice.

[0749] CRISPR/Cas9 and AAV6 were used as above (see for example, Example 3) to create human T cells that lack expression of the TCR, β2M, CD70 with concomitant expression from the TRAC locus using a CAR construct targeting CD70 (SEQ ID NO: 43; SEQ ID NO: 44). In this example activated T cells were first electroporated with 3 distinct Cas9:sgRNA RNP complexes containing sgRNAs targeting TRAC (SEQ ID NO: 40), β2M (SEQ ID NO: 41), and CD70 (SEQ ID NO: 36 or 37). The DNA double stranded break at the TRAC locus was repaired by homology directed repair with an AAV6-delivered DNA template (SEQ ID NO: 43; SEQ ID NO: 44) (encoding anti-CD70 CAR comprising the amino acid sequence of SEQ ID NO: 45) containing right and left homology arms to the TRAC locus flanking a chimeric antigen receptor cassette (−/+ regulatory elements for gene expression).

[0750] The resulting modified T cells are 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ T cells. The ability of these anti-CD70 CAR+ T cells to ameliorate disease caused by a CD70+ T-cell lymphoma cell line was evaluated in NOG mice using methods employed by Translational Drug Development, LLC (Scottsdale, AZ). In brief, 12, 5-8 week old female, CIEA NOG (NOD.Cg-Prkdc.sup.scidIl2rg.sup.tm1Sug/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. Mice received a subcutaneous inoculation of 3×10.sup.6 HuT78 T-cell lymphoma cells in the right hind flank. When mean tumor size reached 25-75 mm.sup.3 (target of ~50 mm.sup.3), the mice were further divided into 2 treatment groups as shown in Table 26. On Day 1, treatment group 2 received a single 200 µl intravenous dose of anti-CD70 CAR+ T cells according to Table 26.

TABLE-US-00026 TABLE 26 Treatment groups T cell HuT78 treatment Group CAR-T cells (i.v.) N 1 None 3 × 10.sup.6 None 5 cells/mouse 2 3X KO (CD70,) 3 × 10.sup.6 1 × 10.sup.7 4 anti-CD70 CAR+ T cells cells/mouse cells/mouse

[0751] Tumor volume was measured 2 times weekly from day of treatment initiation. By Day 12 post-injection, tumors treated with anti-CD70 CAR T cells began to show a decrease in tumor volume in all treated mice. Treatment with anti-CD70 CAR+ T cells effectively reduced the size of the CD70+ T-cell lymphoma tumors, in all mice tested at Day 15 (FIG. **42**C). These data demonstrate that 3×KO (TRAC−/β2M−/CD70−) anti-CD70 CAR+ cells induce regression of human CD70+ T-cell lymphoma tumors in vivo, with potent activity against established HuT78 T-cell lymphoma xenografts.

TABLE-US-00027 Summary of Sequences SEQ ID NO Description Sequence 1 TRAC Indel AAGAGCAACAAATCTGACT 2 TRAC Indel AAGAGCAACAGTGCTGTGCCTGGAGCAACAAATCTGACTAAGAGCA ACAAATCTGACT 3 TRAC Indel AAGAGCAACAGTGCTGGAGCAACAAATCTGACTAAGAGCAACAAAT CTGACT 4 TRAC Indel AAGAGCAACAGTGCCTGGAGCAACAAATCTGACTAAGAGCAACAAA TCTGACT 5 TRAC Indel AAGAGCAACAGTGCTGACTAAGAGCAACAAATCTGACT 6 TRAC Indel AAGAGCAACAGTGCTGTGGGCCTGGAGCAACAAATCTGACTAAGAG CAACAAATCTGACT 7 TRAC Indel AAGAGCAACAGTGCTGGCCTGGAGCAACAAATCTGACTAAGAGCAA CAAATCTGACT

8 TRAC Indel AAGAGCAACAGTGCTGTGTGCCTGGAGCAACAAATCTGACTAAGAG CAACAAATCTGACT 9 B2M Indel CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGCCTGGAGGCT ATCCAGCGTGAGTCTCTCCTACCCTCCCGCT 10 B2M Indel CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCGCCTGGAGGCTA TCCAGCGTGAGTCTCTCCTACCCTCCCGCT 11 B2M Indel CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGAGGCTATCC AGCGTGAGTCTCTCCTACCCTCCCGCT 12 B2M Indel CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGATAGCCTGG AGGCTATCCAGCGTGAGTCTCTCCTACCCTCCCGCT 13 B2M Indel CGTGGCCTTAGCTGTGCTCGCGCTATCCAGCGTGAGTCTCTCCTACC CTCCCGCT 14 B2M Indel CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGTGGCCTGGAG GCTATCCAGCGTGAGTCTCTCCTACCCTCCCGCT 15 sgRNA nnnnnnnnnnnnnnnnnnnnguuuuagagcuagaaauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugcuuuu 16 sgRNA nnnnnnnnnnnnnnnnnnnnguuuuagagcuagaaauagcaaguuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgagucggugc 17 sgRNA n.sub.(17-30)guuuuagagcuagaaauagcaaguuaaaauaaggcuaguccguuaucaacuugaaa aaguggcaccgagucggugcu.sub.(1-8) 18 4-1BB AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTA nucleotide TGAGACCAGTACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCCG sequence ATTTCCAGAAGAAGAAGAAGGAGGATGTGAACTG 19 4-1BB KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL amino acid sequence 20 CD28 amino SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS acid sequence 21 CD3-z CGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGGCATATCAGCAAG nucleotide GACAGAATCAGCTGTATAACGAACTGAATTTGGGACGCCGCGAGGA sequence GTATGACGTGCTTGATAAACGCCGGGGGAGAGACCCGGAAATGGGG GGTAAACCCCGAAGAAAGAATCCCCAAGAAGGACTCTACAATGAAC TCCAGAAGGATAAGATGGCGGAGGCCTACTCAGAAATAGGTATGAA GGGCGAACGACGACGGGGAAAAGGTCACGATGGCCTCTACCAAGG GTTGAGTACGGCAACCAAAGATACGTACGATGCACTGCATATGCAG GCCCTGCCTCCCAGA 22 CD3-z RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG amino acid KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS sequence TATKDTYDALHMQALPPR 23 CD70 UCACCAAGCCCGCGACCAAUguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E1_T1) 24 CD70 AUCACCAAGCCCGCGACCAAguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E1_T3) 25 CD70 CGGUGCGGCGCAGGCCCUAUguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E1_T4) 26 CD70 GCUUUGGUCCCAUUGGUCGCguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E1_T7) 27 CD70 GCCCGCAGGACGCACCCAUAguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E1_T8) 28 CD70 GUGCAUCCAGCGCUUCGCACguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E1_T10) 29 CD70 CAGCUACGUAUCCAUCGUGAguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU (E3_T1) 30 TRAC AGAGCAACAGUGCUGUGGCCguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU 31 β2M

GCUACUCUCUCUUUCUGGCCguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA
uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU 32 PD-1
CUGCAGCUUCUCCAACACAUguuuuagagcuagaaauagcaaguuaaaauaaggcuag sgRNA
uccguuaucaacuugaaaaaguggcaccgagucggugcUUUU 33 CD70
U*C*A*CCAAGCCCGCGACCAAUguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E1_T1) 34 CD70
A*U*C*ACCAAGCCCGCGACCAAguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E1_T3) 35 CD70
C*G*G*UGCGGCGCAGGCCCUAUguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E1_T4) 36 CD70
G*C*U*UUGGUCCCAUUGGUCGCguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E1_T7) 37 CD70
G*C*C*CGCAGGACGCACCCAUAguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E1_T8) 38 CD70
G*U*G*CAUCCAGCGCUUCGCACguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E1_T10) 39 CD70
C*A*G*CUACGUAUCCAUCGUGAguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U (E3_T1) 40 TRAC
A*G*A*GCAACAGUGCUGUGGCCguuuuagagcuagaaauagcaaguuaaaauaagg sgRNA
cuaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U 41 β2M
G*C*U*ACUCUCUCUUUCUGGCCguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U 42 PD-1
C*U*G*CAGCUUCUCCAACACAUguuuuagagcuagaaauagcaaguuaaaauaaggc sgRNA
uaguccguuaucaacuugaaaaaguggcaccgagucggugcU*U*U*U 43 CD70
CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGC rAAV
GTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCG (CD70B
CAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTGCGGCCGC scFV   with
ACGCGTGAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATAT 41BB)
CGAGTAAACGGTAGTGCTGGGGCTTAGACGCAGGTGTTCTGATTTAT
AGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGAT
AGATTTCCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCT
AATGCCCAGCCTAAGTTGGGGAGACCACTCCAGATTCCAAGATGTA
CAGTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTACTCTGCCA
GAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGA
ATAAGCAGTATTATTAAGTAGCCCTGCATTTCAGGTTTCCTTGAGTG
GCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTT
GGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCAC
GAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACC
GTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCAT
CTGGACTCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGT
CCTAACCCTGATCCTCTTGTCCCACAGATATCCAGAACCCTGACCCT
GCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCT
GCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAG
GATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGT
CTATGGACTTCAGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACAT
CGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAAC
CGGTGCCTAGAGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTC
GTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATAT
AAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCG
CCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCT

CTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACTGGCT
GCAGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGG
GAGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTG
AGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGG
TGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAGTCTCTAGCCATT
TAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGCAAGATAG
TCTTGTAAATGCGGGCCAAGATCTGCACACTGGTATTTCGGTTTTTG
GGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGTTC
GGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGG
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CGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACC
AGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGGCCCTGCTGCAGGG
AGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAG
TCACCCACACAAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTC
ATGTGACTCCACGGAGTACCGGGCGCCGTCCAGGCACCTCGATTAG
TTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTT
TTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGTT
AGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTT
GAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAG
TTTTTTTCTTCCATTTCAGGTGTCGTGACCACCATGGCGCTTCCGGTG
ACAGCACTGCTCCTCCCCTTGGCGCTGTTGCTCCACGCAGCAAGGCC
GCAGGTCCAGTTGGTGCAAAGCGGGGCGGAGGTGAAAAAACCCGG
CGCTTCCGTGAAGGTGTCCTGTAAGGCGTCCGGTTATACGTTCACGA
ACTACGGGATGAATTGGGTTCGCCAAGCGCCGGGGCAGGGACTGAA
ATGGATGGGGTGGATAAATACCTACACCGGCGAACCTACATACGCC
GACGCTTTTAAAGGGCGAGTCACTATGACGCGCGATACCAGCATAT
CCACCGCATACATGGAGCTGTCCCGACTCCGGTCAGACGACACGGC
TGTCTACTATTGTGCTCGGGACTATGGCGATTATGGCATGGACTACT
GGGGTCAGGGTACGACTGTAACAGTTAGTAGTGGTGGAGGCGGCAG
TGGCGGGGGGGGAAGCGGAGGAGGGGGTTCTGGTGACATAGTTATG
ACCCAATCCCCAGATAGTTTGGCGGTTTCTCTGGGCGAGAGGGCAA
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TTTATGCATTGGTACCAGCAAAAACCCGGACAACCGCCGAAGCTGC
TGATCTACTTGGCTTCAAATCTTGAGTCTGGGGTGCCGGACCGATTT
TCTGGTAGTGGAAGCGGAACTGACTTTACGCTCACGATCAGTTCACT
GCAGGCTGAGGATGTAGCGGTCTATTATTGCCAGCACAGTAGAGAA
GTCCCCTGGACCTTCGGTCAAGGCACGAAAGTAGAAATTAAAAGTG
CTGCTGCCTTTGTCCCGGTATTTCTCCCAGCCAAACCGACCACGACT
CCCGCCCCGCGCCCTCCGACACCCGCTCCCACCATCGCCTCTCAACC
TCTTAGTCTTCGCCCCGAGGCATGCCGACCCGCCGCCGGGGGTGCTG
TTCATACGAGGGGCTTGGACTTCGCTTGTGATATTTACATTTGGGCT
CCGTTGGCGGGTACGTGCGGCGTCCTTTTGTTGTCACTCGTTATTACT
TTGTATTGTAATCACAGGAATCGCAAACGGGGCAGAAAGAAACTCC
TGTATATATTCAAACAACCATTTATGAGACCAGTACAAACTACTCAA
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GAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATATCGAGTA LHA   to
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GGGCAGGGACTGAAATGGATGGGGTGGATAAATACCTACACCGGCG 41BB)
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MALPVTALLLPLALLLHAARPQVQLVQSGAEVKKPGASVKVSCKASG amino   acid
YTFTNYGMNWVRQAPGQGLKWMGWINTYTGEPTYADAFKGRVTMT sequence
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GGGGSGGGGSGGGGSGDIVMTQSPDSLAVSLGERATINCRASKSVSTSG scFV   with
YSFMHWYQQKPGQPPKLLIYLASNLESGVPDRFSGSGSGTDFTLTISSLQ 41BB)
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DIVMTQSPDSLAVSLGERATINC<u>RASKSVSTSGYSFMH</u>WYQQKPGQPP scFv   amino
KLLIYLASNLESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQHSREV acid
PWTFGQGTKVEIK<u>GGGGSGGGGSGGGGSG</u>QVQLVQSGAEVKKPGASV sequence
KVSCKASGYTFTNYGMNWVRQAPGQGLKWMGWINTYTGEPTYADAF (linker
<u>KGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARDYGDYGMDYWGQ</u> underlined)
GTTVTVSS 49 CD70B
CAGGTCCAGTTGGTGCAAAGCGGGGCGGAGGTGAAAAAACCCGGCG scFv
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QVQLVQSGAEVKKPGASVKVSCKASGYTFTNYGMNWVRQAPGQGLK scFv   amino
WMGWINTYTGEPTYADAFKGRVTMTRDTSISTAYMELSRLRSDDTAV acid
YYCARDYGDYGMDYWGQGTTVTVSS<u>GGGGSGGGGSGGGGGSG</u>DIVMT sequence
QSPDSLAVSLGERATINCRASKSVSTSGYSFMHWYQQKPGQPPKWYL (linker
ASNLESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQHSREVPWTFG underlined)
QGTKVEIK 51 CD70   VH
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YYCARDYGDYGMDYWGQGTTVTVSS 52 CD70   VL
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PWTFGQGTKVEIK 53 Linker GGGGSGGGGSGGGGGSG 54 BCMA
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RFSEVPARFSGSGSGTDFTLTISSVESEDFAVYYCSQTSHIPYTFGGGTKL underlined) EIK 60
BCMA   VH QVQLVQSGAELKKPGASVKVSCKASGNTLTNYVIHWVRQAPGQRLEW
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62 CD70   VL RASKSVSTSGYSFMH CDR1 (Kabat) 63 CD70   VL <u>SKSVSTSGYSF</u> CDR1
(Chothia) 64 CD70   VL LASNLES CDR2 (Kabat) 65 CD70   VL LAS CDR2 (Chothia) 66
CD70   VL QHSREVPWT CDR3 (Kabat) 67 CD70   VL SREVPW CDR3 (Chothia) 68 CD70
VH NYGMN CDR1 (Kabat) 69 CD70   VH GYTFTNYGMN CDR1 (Chothia) 70 CD70   VH
WINTYTGEPTYADAFKG CDR2 (Kabat) 71 CD70   VH NTYTGE CDR2 (Chothia) 72 CD70
VH DYGDYGMDY CDR3 (Kabat) 73 CD70   VH CARDYGDYGMDYWG CDR3 (Chothia) 74
BCMA   VL RASQSLVHSNGNTHLH CDR1 (Kabat) 75 BCMA   VL RASQSLVHSNGNTHLH
CDR1 (Chothia) 76 BCMA   VL SVSNR CDR2 (Kabat) 77 BCMA   VL SVSNR CDR2
(Chothia) 78 BCMA   VL SQTSHIPYT CDR3 (Kabat) 79 BCMA   VL SQTSHIPYT CDR3
(Chothia) 80 BCMA   VH NYVIH CDR1 (Kabat) 81 BCMA   VH GNTLTNY CDR1 (Chothia)
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(Chothia) 84 BCMA   VH WDWDGFFDP CDR3 (Kabat) 85 BCMA   VH WDWDGFFDP CDR3
(Chothia) 86 TRAC AGAGCAACAGTGCTGTGGCC target sequence 87 anti-CD33
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AUCACCAAGCCCGCGACCAA sgRNA (E1_T3) spacer 93 CD70
CGGUGCGGCGCAGGCCCUAU sgRNA (E1_T4) spacer 94 CD70
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CAGCUACGUAUCCAUCGUGA sgRNA (E3_T1) spacer 98 TRAC
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spacer 100 PD-1 CUGCAGCUUCUCCAACACAU sgRNA spacer 101 CD70
U*C*A*CCAAGCCCGCGACCAAU sgRNA (E1_T3) spacer 102 CD70
A*U*C*ACCAAGCCCGCGACCAA sgRNA (E1_T4) spacer 103 CD70
C*G*G*UGCGGCGCAGGCCCUAU sgRNA (E1_T7) spacer 104 CD70
G*C*U*UUGGUCCCAUUGGUCGC sgRNA (E1_T8) spacer 105 CD70
G*C*C*CGCAGGACGCACCCAUA sgRNA (E1_T10) spacer 106 CD70

G*U*G*CAUCCAGCGCUUCGCAC sgRNA (E3_T10) spacer 107 CD70

C*A*G*CUACGUAUCCAUCGUGA sgRNA (E1_T3) spacer 108 TRAC

A*G*A*GCAACAGUGCUGUGGCC spacer 109 β2M G*C*U*ACUCUCUCUUUCUGGCC

sgRNA spacer 110 PD-1 C*U*G*CAGCUUCUCCAACACAU sgRNA spacer 111 CD70

TCACCAAGCCCGCGACCAAT<u>GGG</u> sgRNA (E1_T1) with   PAM 112 CD70

ATCACCAAGCCCGCGACCAA<u>TGG</u> sgRNA (E1_T3) with   PAM 113 CD70

CGGTGCGGCGCAGGCCCTAT<u>GGG</u> sgRNA (E1_T4) with   PAM 114 CD70

GCTTTGGTCCCATTGGTCGC<u>GGG</u> sgRNA (E1_T7) with   PAM 115 CD70

GCCCGCAGGACGCACCCATA<u>GGG</u> sgRNA (E1_T8) with   PAM 116 CD70

GTGCATCCAGCGCTTCGCAC<u>AGG</u> sgRNA (E1_T10) with   PAM 117 CD70

CAGCTACGTATCCATCGTGA<u>TGG</u> sgRNA (E3_T1) with   PAM 118 TRAC

AGAGCAACAGTGCTGTGGCC<u>TGG</u> sgRNA   with PAM 119 β2M

GCTACTCTCTCTTTCTGGCC<u>TGG</u> sgRNA   with PAM 120 PD-1

CTGCAGCTTCTCCAACACAT<u>CGG</u> sgRNA   with PAM 121 CD28

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PRLLIY**WASTRES**GVPDRFTGSGSGTDFTLTISSVQPEDLAIYYC**HQYLS** VL
SRTFGQGTKLEIK CDRs underlined and   in   bold 142 anti-CD33 <u>SYYIH</u> antibody VH
CDR1 (Kabat) 143 anti-CD33 <u>VIYPGNDDISYNQKFQG</u> antibody′ VH   CDR2 (Kabat) 144 anti-
CD33 <u>EVRLRYFDV</u> antibody VH   CDR3 (Kabat) 145 anti-CD33 <u>KSSQSVFFSSSQKNYLA</u>

antibody VL   CDR1 (Kabat   & Chothia) 146 anti-CD33 <u>WASTRES</u> antibody VL   CDR2 (Kabat   & Chothia 147 anti-CD33 <u>HQYLSSRT</u> antibody VL   CDR3 (Kabat   & Chothia) 148 Anti-CD19 ATGCTTCTTTTGGTTACGTCTCTGTTGCTTTGCGAACTTCCTCATCCA CAR GCGTTCTTGCTGATCCCCGATATTCAGATGACTCAGACCACCAGTAG CD8[tm]-CTTGTCTGCCTCACTGGGAGACCGAGTAACAATCTCCTGCAGGGCAA CD28   [co-GTCAAGACATTAGCAAATACCTCAATTGGTACCAGCAGAAGCCCGA stimulatory CGGAACGGTAAAACTCCTCATCTATCATACGTCAAGGTTGCATTCCG domain]-GAGTACCGTCACGATTTTCAGGTTCTGGGAGCGGAACTGACTATTCC CD3z) TTGACTATTTCAAACCTCGAGCAGGAGGACATTGCGACATATTTTTG TCAACAAGGTAATACCCTCCCTTACACTTTCGGAGGAGGAACCAAA CTCGAAATTACCGGGTCCACCAGTGGCTCTGGGAAGCCTGGCAGTG GAGAAGGTTCCACTAAAGGCGAGGTGAAGCTCCAGGAGAGCGGCCC CGGTCTCGTTGCCCCCAGTCAAAGCCTCTCTGTAACGTGCACAGTGA GTGGTGTATCATTGCCTGATTATGGCGTCTCCTGGATAAGGCAGCCC CCGCGAAAGGGTCTTGAATGGCTTGGGGTAATATGGGGCTCAGAGA CAACGTATTATAACTCCGCTCTCAAAAGTCGCTTGACGATAATAAAA GATAACTCCAAGAGTCAAGTTTTCCTTAAAATGAACAGTTTGCAGAC TGACGATACCGCTATATATTATTGTGCTAAACATTATTACTACGGCG GTAGTTACGCGATGGATTATTGGGGGCAGGGGACTTCTGTCACAGTC AGTAGTGCTGCTGCCTTTGTCCCGGTATTTCTCCCAGCCAAACCGAC CACGACTCCCGCCCCGCGCCCTCCGACACCCGCTCCCACCATCGCCT CTCAACCTCTTAGTCTTCGCCCCGAGGCATGCCGACCCGCCGCCGGG GGTGCTGTTCATACGAGGGGCTTGGACTTCGCTTGTGATATTTACAT TTGGGCTCCGTTGGCGGGTACGTGCGGCGTCCTTTTGTTGTCACTCGT TATTACTTTGTATTGTAATCACAGGAATCGCTCAAAGCGGAGTAGGT TGTTGCATTCCGATTACATGAATATGACTCCTCGCCGGCCTGGGCCG ACAAGAAAACATTACCAACCCTATGCCCCCCCACGAGACTTCGCTGC GTACAGGTCCCGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGGCA TATCAGCAAGGACAGAATCAGCTGTATAACGAACTGAATTTGGGAC GCCGCGAGGAGTATGACGTGCTTGATAAACGCCGGGGGAGAGACCC GGAAATGGGGGGTAAACCCCGAAGAAAGAATCCCCAAGAAGGACT CTACAATGAACTCCAGAAGGATAAGATGGCGGAGGCCTACTCAGAA ATAGGTATGAAGGGCGAACGACGACGGGGAAAAGGTCACGATGGC CTCTACCAAGGGTTGAGTACGGCAACCAAAGATACGTACGATGCAC TGCATATGCAGGCCCTGCCTCCCAGA 149 Anti-CD19 MLLLVTSLLLCELPHPAFLLIPDIQMTQTTSSLSASLGDRVTISCRASQDI CAR SKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNL CD8[tm]-EQEDIATYFCQQGNTLPYTFGGGTKLEITGSTSGSGKPGSGEGSTKGEV CD28   [co-KLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVI stimulatory WGSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY domain]-YYGGSYAMDYWGQGTSVTVSSAAAFVPVFLPAKPTTTPAPRPPTPAPTI CD3z) ASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLV Amino   Acid ITLYCNHRNRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY RSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEM GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR 150 Anti-CD19 GATATTCAGATGACTCAGACCACCAGTAGCTTGTCTGCCTCACTGGG scFv AGACCGAGTAACAATCTCCTGCAGGGCAAGTCAAGACATTAGCAAA TACCTCAATTGGTACCAGCAGAAGCCCGACGGAACGGTAAAACTCC TCATCTATCATACGTCAAGGTTGCATTCCGGAGTACCGTCACGATTT

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[0752] All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

[0753] The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

[0754] It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

[0755] In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, i.e., to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

[0756] The terms "about" and "substantially" preceding a numerical value mean±10% of the recited numerical value.

[0757] Where a range of values is provided, each value between the upper and lower ends of the range are specifically contemplated and described herein.

Claims

1. An engineered T cell comprising a disrupted CD70 gene and a nucleic acid encoding a chimeric antigen receptor (CAR) that does not bind CD70.

2. The engineered T cell of claim 1, further comprising a disrupted T cell receptor alpha constant region (TRAC) gene.

3. The engineered T cell of claim 1, further comprising a disrupted beta-2-microglobulin (β2M) gene.

4. The engineered T cell of claim 1, wherein the disrupted TRAC gene comprises the nucleic acid encoding the CAR.

5. The engineered T cell of claim 1, wherein the CAR comprises an ectodomain that binds anti-B cell maturation antigen (BCMA).

6. The engineered T cell of claim 5, wherein the ectodomain comprises an anti-BCMA antibody.

7. The engineered T cell of claim 5, wherein the ectodomain comprises an anti-BCMA single-chain variable fragment (scFv).

8. The engineered T cell of claim 7, wherein the anti-BCMA scFv comprises variable heavy (VH) chain complementarity determining regions (CDRs) and the same variable light (VL) chain CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 60 and a VL set forth as SEQ ID NO: 61.

9. The engineered T cell of claim 7, wherein the anti-BCMA scFv comprises VH and VL chains comprising the amino acid sequences set forth in SEQ ID NOs: 60 and 61, respectively.

10. The engineered T cell of claim 7, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 59.

11. The engineered T cell of claim 1, wherein the CAR comprises an ectodomain that binds CD33.

12. The engineered T cell of claim 11, wherein the ectodomain comprises an anti-CD33 antibody.

13. The engineered T cell of claim 11, wherein the ectodomain comprises an anti-CD33 scFv.

14. The engineered T cell of claim 13, wherein the anti-CD33 scFv comprises the same VH CDRs and the same VL chain CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 140 and a VL set forth as SEQ ID NO: 141.

15. The engineered T cell of claim 11, wherein the anti-CD33 scFv comprises VH and VL chains comprising the amino acid sequences set forth in SEQ ID NOs: 140 and 141, respectively.

16. The engineered T cell of claim 11, wherein the anti-CD33 scFv comprises the amino acid sequence of SEQ ID NO: 137.

17. The engineered T cell of claim 1, wherein the CAR comprises an ectodomain that binds CD19.

18. The engineered T cell of claim 17, wherein the ectodomain comprises an anti-CD19 antibody.

19. The engineered T cell of claim 17, wherein the ectodomain comprises an anti-CD19 scFv.

20. The engineered T cell of claim 19, wherein the anti-CD19 scFv comprises the same VH CDRs and the same VL chain CDRs as a reference antibody, wherein the reference antibody comprises a VH set forth as SEQ ID NO: 152 and a VL set forth as SEQ ID NO: 153.

21. (canceled)

22. (canceled)

23. (canceled)